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Optimizing Avian Fertility: Caffeine Modulation of Sperm Motility in Red Junglefowl

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Abstract

The Red Junglefowl (RJF; *Gallus gallus*) represents the wild ancestor of domesticated chickens yet its numbers experience reduction because of habitat destruction together with hunting practices and mixed gene flow. The improvement of reproductive biotechnology is an essential requirement for conservation initiatives. The study tested how caffeine impacts sperm motility together with kinematic parameters in RJF semen through CASA. Semen samples were collected from 20 mature RJF males then diluted using increasing caffeine levels (1, 2, 3 and 4 mg/ml) while maintaining a control group with 0 mg/ml of extender. The study determined sperm total motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) at 0, 30 and 60 minutes. The sperm motility increased significantly when caffeine was added at 2 mg/ml concentration because total and progressive motility reached maximum levels ($P<0.05$) at 30 minutes. The specific values for VCL, VSL and VAP reached at the best levels with this level (2 mg/ml). The application of 4 mg/ml caffeine led to decreased sperm movement and raised mortality rates yet revealing harmful effects. These results demonstrate how proper concentrations of caffeine function as performance-enhancing agents that efficiently boost sperm motility in RJF. The appropriate levels of caffeine becomes essential because it displays harmful effects against sperm viability. It is necessary to conduct additional investigations about the sustained impact of caffeine on sperm capabilities alongside its role in semen preservation through freezing methods. The study contributes significant knowledge about using caffeine for research in avian reproductive biotechnology.

Keywords: Caffeine; sperm motility; semen extenders; Red junglefowl; conservation breeding.

Introduction

The Red Junglefowl (RJF; *Gallus gallus*) is well known as the natural ancestor of domestic chickens and stands vital for both poultry breeding and genetic conservation (Eriksson *et al*., 2008). The population of RJF faces severe decline because of habitat loss and mating between wild and domesticated chickens and excessive hunting put it under serious risk (Singchat *et al*., 2022). Genetic diversity deterioration threatens both biodiversity and poultry breeding program sustainability (Uddin *et al*., 2011). Strategies for effective conservation must be established because they serve to protect this species' genetic makeup and stop its population from continuing to decrease.

As stated by Peters *et al*. (2008) reproductive efficiency serves as one of the essential factors to achieve conservation success in birds and sperm motility directly impacts fertility outcomes. The capability of sperms to move through the female reproductive tract depends on motility because this trait enables successful fertilization during artificial insemination (AI) and breeding procedures (McGary *et al*., 2002). The assessment of semen quality includes analysis

of total motility along with measurements of progressive motility and viability as well as multiple kinematic parameters according to Chakraborty and Saha (2022). Scant information exists about reproductive traits in RJF due to the scarce research on this wild chicken species (Rahman *et al*., 2021).

Research teams have investigated the use of caffeine-based pharmacological agents for improving sperm motility and fertility levels. The natural stimulant called Caffeine (1,3,7-trimethylxanthine) occurs naturally in coffee beans and tea leaves as well as cocoa beans. Scientific reports show that sperm motility is increased through caffeine exposure because of the rising of intracellular cyclic adenosine monophosphate (cAMP) levels (Nassar *et al*., 1998). Caffeine functions by blocking phosphodiesterase enzymes to promote cAMP accumulation then enhances both mitochondrial energy generation and sperm motility and ATP synthesis (Agarwal and Prabakaran, 2005). Caffeine supplementation shows favorable effects on sperm motion without any negative impact on survival rates according to studies on mammals and birds (Parkhurst *et al*., 2000). Turkeys along with other species have been demonstrated that caffeine enhances both sperm motility and fertilization success (Yamaguchi *et al*., 2009). After exposure with caffeine bovine and human sperms showed greater acrosome reaction activity which is fundamental for fertilization (Taylor *et al*., 2013). The research indicates that caffeine functions as a metabolic enhancer because it stimulates mitochondria and increases sperm motility which leads to higher fertility rates (Valverde *et al*., 2019). Few studies have investigated the effects of caffeine on spermatozoa from the endangered RJF species though scientists have thoroughly analyzed this topic for mammals and domestic poultry.

Research on caffeine treatment as an approach to improve semen quality in RJF becomes crucial because of their genetic importance and endangered status. Discovery of the best caffeine level to enhance sperm movement capability with preserved viability would substantially advance artificial breeding strategies for this endangered species. Research on the biochemical terms alongside molecular processes through which caffeine affects sperm motility could lead to better comprehension of avian reproductive functions. Therefore, the present study investigated the connection by analyzing how caffeine affects motility and kinematic parameters of RJF sperm using CASA technology. Research optimization of caffeine concentrations will give important data to enhance artificial breeding and genetic conservation methods for RJF populations throughout the globe.

Materials and Methods

Study Location and Duration

This study was conducted at the Advanced Avian Research Farm, and Genetics and Animal Breeding Laboratory, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh, between February and July 2023.

Experimental Birds and Management

A total of 20 mature male RJFs, average live weight was 1.8 kg, were used for this study. The birds were kept in an intensive management system with a 16-hour light schedule. Each bird received 100 g/day of a commercially available breeder diet and had *ad libitum* access to fresh water. The housing conditions were designed to ensure minimal stress, thereby optimizing reproductive efficiency.

The birds were housed individually in well-ventilated cages to prevent aggressive interactions, a common concern among male RJFs. Cages were equipped with perches and nest boxes to provide a natural environment and reduce stress-induced behavioral abnormalities. Regular health checks were conducted to monitor the well-being of the birds, including weight assessments, feather condition evaluations, and screening for common avian diseases. Biosecurity measures, such as restricted access to housing facilities and periodic disinfection, were strictly enforced to minimize the risk of infections.

To simulate natural mating behaviors and improve semen quality, auditory and visual stimuli, such as recorded RJF calls and mirrors, were introduced. These enrichment techniques have been reported to enhance libido and increase sperm production in various avian species (Small *et al*., 2008; Mitoyen *et al*., 2019).

Semen Collection and Processing

Semen was collected twice weekly using the standard abdominal massage technique described by Burrows and Quinn (1937). Birds were trained for semen collection over four weeks before the study commenced to ensure optimal semen quality and minimize handling stress. Each collection was performed in the morning (9:00–10:00 AM) to ensure consistency in sperm quality. The birds were gently restrained, and the cloacal region was massaged with a standardized technique to stimulate ejaculation. Semen was collected in sterile and pre-warmed (37°C) 1 ml microtubes to prevent cold shock, which can negatively impact sperm viability. Special care was taken to avoid contamination with feces, feathers, or other debris. After collection, semen samples were evaluated immediately for volume, color, and contamination under a stereomicroscope. Any samples exhibiting abnormalities such as blood contamination or excessive debris were discarded. The samples were then maintained in insulated containers at 37°C during transportation to the laboratory, ensuring that temperature fluctuations did not affect sperm

quality. Upon arrival at the laboratory, semen was subjected to initial quality assessments, including sperm concentration using a hemocytometer and viability evaluation through eosin-nigrosin staining. Samples with sperm motility below 60% were excluded from further analysis. The semen was then diluted in modified Ringer's solution before subsequent treatments and analysis.

Semen Dilution and Experimental Design

The collected semen was diluted using modified Ringer's solution, which contained essential electrolytes and energy sources necessary for sperm viability and motility. The composition of the Ringer's solution was as follows: 9.50 g Sodium chloride, 0.20 g Potassium chloride, 0.26 g Calcium chloride, 0.20 g Sodium bicarbonate, 1.00 g Glucose, and 1 liter of distilled water (Akcay *et al*., 2006).

The diluted semen samples were then allocated into five experimental treatment groups T0: (Control) No caffeine (0 mg/ml); T1: 1 mg/ml caffeine; T2: 2 mg/ml caffeine; T3: 3 mg/ml caffeine and T4: 4 mg/ml caffeine. Each sample was incubated at 37°C for up to 60 minutes, ensuring optimal sperm activity during the analysis period. Semen samples were gently mixed before assessments to ensure uniform distribution of spermatozoa in the solution. Assessments were conducted at three specific time intervals: 0 (Baseline assessment), 30 (Midpoint assessment) and 60 minutes (Final assessment). Throughout the incubation period, samples were maintained in controlled conditions to avoid exposure to temperature fluctuations or external contaminants. Repeated measures were taken to assess the progressive impact of caffeine concentrations on sperm motility and kinetic parameters over time. Semen samples were carefully analyzed using Computer Assisted Semen Analysis (CASA), a sophisticated and highly accurate system that allows for the detailed assessment of sperm motility and kinematic parameters. This technique is essential for understanding the functional aspects of sperm, especially in evaluating fertility potential.

For the analysis, semen aliquots were diluted at a 1:20 ratio using a modified Ringer's solution This dilution ratio was optimized for CASA analysis (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 motility and kinematic parameters were analyzed using the CASA system fitted with an avian-specific condenser (ph-1) and a 10× objective, which assessed key aspects of sperm movement. To maintain statistical significance and provide a comprehensive assessment of the sample, at least 500 sperms per sample were counted as this large sample size enhances the reliability and robustness of the results.

Among the kinematic parameters, several velocity parameters were analyzed to characterize sperm movement. Curvilinear velocity (VCL) represented the actual speed of sperm along its curved path, while straight-line velocity (VSL) measured the speed between its initial and final position, indicating the efficiency of direct movement. Average path velocity (VAP) provided an integrated measure of both linear and non-linear movement, reflecting overall swimming behavior. Additional parameters were also examined to assess movement patterns. Linearity (LIN) and straightness (STR) evaluated the directionality and efficiency of sperm movement, with higher values indicating more direct trajectories. Wobble (WOB) measured the degree of lateral deviation, with higher values suggesting erratic movement. The amplitude of lateral head displacement (ALH) quantified side-to-side movement of the sperm head, with excessive displacement indicating abnormal motility. Lastly, beat cross frequency (BCF) captured the frequency of tail beats, which is associated with the vigor and energy of sperm motility.

For this analysis, the SCA motility software (Microptic Automatic Diagnostic System, Barcelona, Spain) was employed. This software was specifically configured for avian species, enabling the precise measurement of sperm motion based on their unique characteristics. The system used a 10X negative phase contrast objective lens for object analysis, allowing for clear and detailed observation of sperm, enhancing the accuracy of the motility measurements.

Overall, CASA is an essential tool in sperm quality analysis, providing a high level of precision and reliability in evaluating sperm motility and its associated kinematic parameters. This data is invaluable in both clinical and research settings, particularly when studying fertility, reproductive health, and the effects of various treatments or interventions.

Statistical Analysis

The data for sperm concentration, motility, progressive motility, sperm kinetics (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF), fertility, hatchability, embryonic mortality, day-old poult weight, and survivability 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 treatment 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.

Results and Discussion

Total Sperm Motility

The results regarding the total sperm motility were demonstrated in Fig. 1. The supplementation of caffeine has

a significant impact on sperm motility in Red Junglefowl (RJFs), with the highest motility observed in the 2 mg/ml caffeine-treated group (T2). Total sperm motility increased at 30 minutes, followed by a decline at 60 minutes, suggesting a transient stimulatory effect of caffeine. This finding aligns with previous research indicating that caffeine enhances total sperm motility by increasing intracellular cyclic adenosine monophosphate (cAMP) levels, which subsequently activate protein kinase A (PKA) and enhance flagellar motion (Nassar *et al*., 1998; Yamaguchi *et al*., 2009). However, the decline observed at 60 minutes suggests that prolonged exposure may reduce efficacy, possibly due to ATP depletion or oxidative stress, as reported in other avian species (Parkhurst *et al*., 2000).

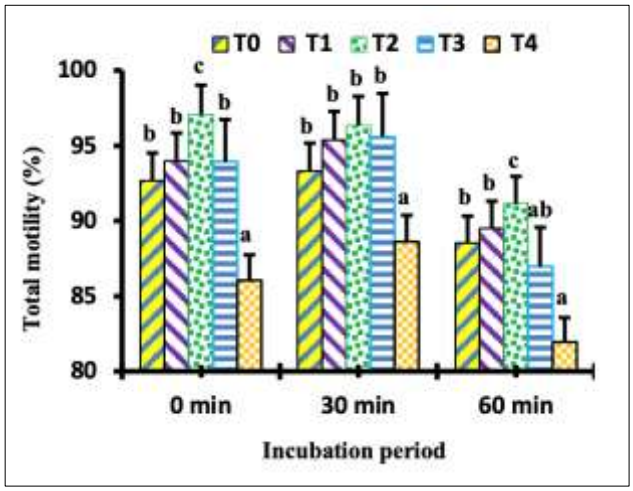

Fig. 1: Effect of caffeine supplementation on total sperm motility (%) at different incubation periods in Red Junglefowl. Each bar with error bar represents mean±SEM value. Different letters on error bar indicate significant differences (P<0.05) among the treatment groups: T0: (Control, no caffeine, 0 mg/ml); T1: 1 mg/ml caffeine; T2: 2 mg/ml caffeine; T3: 3 mg/ml caffeine and T4: 4 mg/ml caffeine

The dose-dependent effects of caffeine on motility suggest that moderate levels optimize energy utilization, while excessive caffeine may induce cellular stress. This is consistent with studies on mammalian sperm, where caffeine has been shown to transiently enhance motility before eventual metabolic exhaustion due to prolonged stimulation (Agarwal and Prabakaran, 2005). These findings highlight the importance of controlled caffeine exposure to maximize its benefits while avoiding potential toxicity.

Progressive Motility

Effect of different levels of caffeine on progressive motility at different incubation periods are shown in Fig. 2. The results also followed a similar trend with the highest values recorded in the T2 group. Progressive motility is critical for fertilization success, as it determines the sperm's ability to travel efficiently toward the egg (McGary *et al*., 2002). The enhancement in progressive motility observed in this study suggests that caffeine improves sperm functionality, consistent with findings in mammalian species where caffeine has been shown to increase hyperactivation and directional movement (Iorio *et al*., 2020). However, at higher concentrations (T4, 4 mg/ml), progressive motility significantly decreased, indicating a potential toxic effect at excessive caffeine levels.

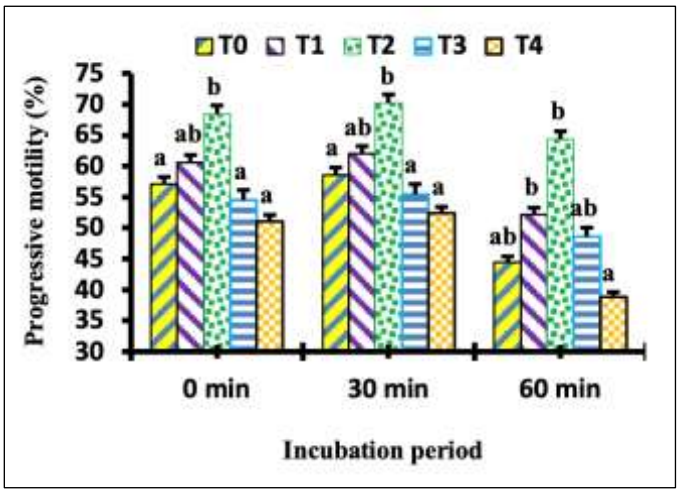

Fig. 2: Effect of caffeine supplementation on progressive motility (%) at different incubation periods in Red Junglefowl. Each bar with error bar represents mean±SEM value. Different letters on error bar indicate significant differences (P<0.05) among the treatment groups: T0: (Control, no caffeine, 0 mg/ml); T1: 1 mg/ml caffeine; T2: 2 mg/ml caffeine; T3: 3 mg/ml caffeine and T4: 4 mg/ml caffeine

The observed improvements in progressive motility could be attributed to caffeine's role in calcium channel activation, which enhances flagellar beating patterns and forward progression (Nabavi *et al*., 2013). However, excessive calcium influx at higher caffeine concentrations may result in premature capacitation-like changes, leading to reduced motility and viability. This phenomenon has been observed in studies on human spermatozoa, where high caffeine levels led to excessive calcium influx, causing hyperactivation followed by motility loss (Valverde *et al*., 2019). Further research is needed to clarify whether similar mechanisms apply to avian sperm.

Kinematic Parameters

Table 1 demonstrates that the sperm kinematic parameters, including curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL), were highest in the T2 group at 30 minutes, with a subsequent decline at 60 minutes. These findings corroborate studies in mammalian species, where caffeine has been shown to enhance sperm velocity and movement efficiency (Valverde *et al*., 2019). The observed increase in VCL, VAP, and VSL suggests improved energy utilization, likely due to caffeine's role in stimulating mitochondrial ATP production (Agarwal and

Prabakaran, 2005). However, the reduction in these parameters at higher caffeine concentrations (T3 and T4) suggests that excessive caffeine may impair mitochondrial function or induce oxidative damage, as observed in previous studies on boar spermatozoa (Yamaguchi *et al*., 2009).

The supplementation of caffeine also influenced straightness (STR), linearity (LIN), and wobble (WOB) across different incubation periods (Table 2). Among all treatment groups, T2 (moderate caffeine level) consistently exhibited the highest values for all parameters, indicating a potential optimal concentration for improving sperm motility. Straightness (STR) was highest in T2 across all time points, suggesting enhanced directional movement. While T1 also showed a slight improvement over the control, T3 and T4 exhibited lower values, particularly at 60 minutes, indicating that excessive caffeine supplementation may have a detrimental effect on sperm trajectory. Linearity (LIN) followed a similar trend, with T2 demonstrating the most pronounced improvement, particularly at 30 minutes (37.00 ± 1.10). This suggests that moderate caffeine levels may enhance the precision of sperm movement. However, a decline in LIN at 60 minutes, especially in T3 and T4, indicates possible caffeine-induced hyperactivation, which can lead to erratic movement and reduced fertilization potential. Wobble (WOB) values were also highest in T2, indicating a balanced motility pattern. The control (T0) and lower caffeine groups (T1) maintained moderate levels, while the highest caffeine doses (T3 and T4) resulted in decreased WOB at 60 minutes, suggesting impaired motility due to excessive stimulation.

Table 1: Effect of caffeine supplementation on sperm velocity (µm/sec) at different incubation periods in red junglefowl

Parameter	Time (min)	T0 (Control)	T1	T2	T3	T4
Curvilinear Velocity	0	94.85 ± 1.25^{a}	95.6 ± 1.32^{a}	104.18 ± 1.45^{b}	92.92 ± 1.21^{a}	89.72 ± 1.10^{a}
	30	95.44 ± 1.28^{a}	96.26 ± 1.35^{a}	106.13 ± 1.50^{b}	93.81 ± 1.23^{a}	90.4 ± 1.12^{a}
	60	90.95 ± 1.15^{a}	91.49 ± 1.20^{a}	97.42 ± 1.30^{b}	88.79 ± 1.10^{a}	86.91 ± 1.05^{a}
Average Path Velocity	0	55.97 ± 1.05^{a}	56.4 ± 1.12^{ab}	60.44 ± 1.15^{b}	53.05± 1.10^{a}	50.5± 1.00^{a}
	30	56.56 ± 1.18^{a}	56.56 ± 1.05^{ab}	61.69 ± 1.20^{b}	53.39 ± 1.03^{a}	51.24 ± 1.02^{a}
	60	51.83 ± 1.05^{a}	52.28 ± 1.10^{ab}	56.03 ± 1.00^{b}	50.6 ± 1.11^{a}	47.28 ± 1.01^{a}
Straight Line Velocity	0	31.39 ± 0.65^{a}	31.6 ± 0.70^{a}	36.23 ± 0.75^{b}	30.64 ± 0.60^{a}	29.61 ± 0.58^{a}
	30	31.96 ± 0.67^{a}	32.22 ± 0.72^{a}	37.3 ± 0.78^{b}	31.03 ± 0.63^{a}	29.61 ± 0.58^{a}
	60	28.63 ± 0.60^{ab}	29.62 ± 0.65^{b}	33.74 ± 0.70^{c}	27.37 ± 0.55^{a}	26.03 ± 0.50^{a}

Values are Means±SEM; ^{a,b}Means within a row without common superscripts differ significantly, where T0: (Control, no caffeine, 0 mg/ml); T1: 1 mg/ml caffeine; T2: 2 mg/ml caffeine; T3: 3 mg/ml caffeine and T4: 4 mg/ml caffeine

Table 2: The straingtness (STR), Linearity (LIN) and Wobble (WOB) percentages at different incubation periods by supplementation of different levels of caffeine

Parameter	Time (min)	T0 (Control)	T1	T2	T3	T4
Straightness (STR)	0	52.3 ± 1.21^{a}	52.64 ± 1.34^{a}	57.57 ± 1.11^{b}	51.71 ± 1.45^{a}	47.8 ± 1.56^{a}
	30	52.09 ± 1.08^{a}	53.38 ± 1.25^{ab}	58.4 ± 1.15^{b}	52.16 ± 1.39^{a}	48.15 ± 1.49^{a}
	60	47.01 ± 1.33^{a}	47.74 ± 1.40^{a}	53.3 ± 1.23^{b}	46.88 ± 1.52^{a}	45.92 ± 1.58^{a}
Linearity (LIN)	0	29.3 ± 0.98^{a}	31.26 ± 1.04^{ab}	35.84 ± 1.01^{ab}	29.7 ± 1.12^{a}	27.05 ± 1.23^{a}
	30	30.07 ± 0.92^{a}	32.00 ± 1.08^{ab}	37.00 ± 1.10^{b}	30.39 ± 1.19^{a}	27.91 ± 1.31^{a}
	60	26.24 ± 1.02^{a}	26.39 ± 1.09^{a}	31.55 ± 1.05^{b}	25.32 ± 1.27^{a}	24.31 ± 1.35^{a}
Wobble (WOB)	0	53.84 ± 1.41^{a}	55.60 ± 1.50^{ab}	62.97 ± 1.35^{b}	54.62 ± 1.58^{ab}	49.27 ± 1.63^{a}
	30	54.53 ± 1.38^{a}	55.91 ± 1.48^{ab}	64.29 ± 1.40^{b}	55.25 ± 1.55^{ab}	49.87 ± 1.71^{a}
	60	50.35 ± 1.45^{a}	50.76 ± 1.52^{a}	58.11 ± 1.47^{b}	48.43 ± 1.66^{a}	47.18 ± 1.73^{a}

Values are Means±SEM; ^{a,b}Means within a row without common superscripts differ significantly, where T0: (Control, no caffeine, 0 mg/ml); T1: 1 mg/ml caffeine; T2: 2 mg/ml caffeine; T3: 3 mg/ml caffeine and T4: 4 mg/ml caffeine

The role of mitochondrial function in sperm motility is crucial, as energy-dependent flagellar movement is directly linked to ATP availability (Taylor *et al*., 2013). Studies have shown that caffeine enhances mitochondrial respiration at moderate doses but can disrupt oxidative phosphorylation at high doses, leading to sperm dysfunction (Yeste *et al*., 2008). The decline in kinematic parameters at higher caffeine levels in this study aligns with this understanding, reinforcing the importance of dose optimization. Overall, these findings indicate that moderate caffeine supplementation enhances sperm motility parameters, while excessive caffeine levels may lead to reduced efficiency. This highlights the importance of optimizing caffeine concentration to maximize reproductive potential without inducing adverse effects.

Sperm Viability and Toxicity

Sperm viability was highest in the T2 group and lowest in the T4 group, indicating that caffeine at moderate levels enhances sperm survival, while excessive concentrations may induce toxicity. The detrimental effects at higher caffeine concentrations may be attributed to increased reactive oxygen species (ROS) production, which damages sperm membranes and reduces viability (Agarwal and Prabakaran, 2005). Similar findings have been reported in poultry species, where excessive caffeine exposure led to increased lipid peroxidation and DNA fragmentation (Taylor *et al*., 2013). These results highlight the importance of precise dosage regulation to optimize sperm quality without inducing cytotoxic effects.

Caffeine-induced ROS generation at higher concentrations has been documented in sperm from multiple species, where excessive oxidative stress leads to lipid peroxidation and DNA fragmentation (Harrison *et al*., 1980). The observed increase in dead spermatozoa in the T4 group may be attributed to such oxidative stress mechanisms. Antioxidant supplementation in conjunction with caffeine has been proposed as a potential strategy to mitigate these adverse effects (Yamaguchi *et al*., 2009), an approach that warrants further investigation in avian species.

Conservation and Practical Applications

The findings of this study have significant implications for the conservation of RJFs. As a species facing genetic dilution and population decline, improving reproductive efficiency through semen extenders can aid breeding programs. The use of caffeine at an optimal concentration (2 mg/ml) could enhance AI success rates, particularly in ex-situ conservation efforts (Hamid *et al*., 2019). Additionally, the potential for caffeine supplementation in semen cryopreservation warrants further investigation, as it may help maintain sperm motility during long-term storage (Yeste *et al*., 2008).

Artificial reproductive technologies are increasingly used for endangered species conservation, and optimizing semen storage conditions is critical (Eriksson *et al*., 2008). Given that RJFs are a key genetic reservoir for domestic chickens, improving semen preservation techniques can benefit poultry breeding programs as well. Further research on cryopreservation protocols incorporating caffeine may provide novel strategies for long-term sperm storage while maintaining functional integrity.

Limitations and Future Directions

While this study provides valuable insights into the effects of caffeine on RJF sperm motility, certain limitations should be considered. First, this study was conducted *in vitro*, and *in vivo* fertilization success of caffeine-treated sperm remains unknown. Future studies should investigate the long-term effects of caffeine on sperm viability, membrane integrity, and fertilization potential. Moreover, exploring the molecular mechanisms underlying caffeine's action on avian spermatozoa could provide deeper insights into its role in sperm function and fertility.

Additionally, dose-dependent effects of caffeine on sperm capacitation, acrosome reaction, and fertilization potential warrant further investigation. Studies on other avian species would help determine whether the observed effects are consistent across different bird species. Moreover, evaluating caffeine's impact on sperm longevity beyond the 60-minute incubation period could provide insights into its practical application in AI and semen storage.

Conclusions

In conclusion, caffeine supplementation at an optimal concentration (2 mg/ml) significantly enhances sperm motility, progressive motility, and kinematic parameters in RJF semen. However, higher concentrations negatively affect sperm viability, highlighting the need for precise dosage regulation. These findings have practical applications in AI and conservation breeding programs, potentially improving reproductive success in RJFs. Further research is required to assess the long-term implications of caffeine use in semen storage and fertilization outcomes, particularly under field conditions. Additionally, investigating antioxidant co-supplementation strategies may help counteract oxidative stress at higher caffeine concentrations, ensuring improved reproductive outcomes in conservation programs.

Authors' Contribution

All authors contributed equally at all stages of research and manuscript preparation. Final form of manuscript was approved by all authors.

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