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Research Article

Evaluating the Cryoprotective Effects of Butylated Hydroxytoluene on Semen Quality Parameters of *Phasianus colchicus*

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ABSTRACT

Cryopreservation is critical for the preservation of genetic resources and the propagation of endangered species. The choice of proper cryoprotectants significantly affects the success of cryopreservation. The current study investigated the impact of butylated-hydroxytoluene (BHT) on sperm microscopic assays in Ring-necked Pheasants at various post-processing phases, including post-dilution, post-cooling, post-equilibration, and post-thawing. Semen samples were collected and cryopreserved at various times with BHT (0.0 mM (control), 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM. Acrosomal integrity (AI), viability, plasma membrane integrity (PMI), and sperm motility were all assessed as significant markers of sperm quality. It was discovered that using a 1.0 mM concentration of BHT had a significant ($p > 0.05$) influence on each stage of cryopreservation, resulting in improvements in sperm motility, PMI, viability, and AI when analyzed in both control and other groups. These findings emphasize BHT's cryoprotective qualities of BHT in protecting the quality and integrity of Ring-necked Pheasant sperm during cryopreservation. Further research is needed to understand the mechanisms by which BHT exerts its protective effects and to maximize its use in assisted reproductive treatments. The use of BHT as a cryoprotectant has the potential to aid in the conservation of endangered bird species, such as the Ring-necked Pheasant.

Keywords: Ring-necked Pheasant, semen, Butylated hydroxytoluene, cryopreservation, freezing.

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INTRODUCTION

The extinction of a population indicates a deficit of a resource that has been subjected to extensive natural selection. Captive breeding, reproductive technologies, and conservation efforts in the natural environment of a species are critical for combating biodiversity extinction (Prieto et al., 2014; Rakha et al., 2013; Blanco et al., 2012; Blesbois et al., 2008; Bilal et al., 2021). Use of this preservation strategy can be expanded to protect the genetic integrity of these important subspecies (Rakha et al., 2015, 2016a,b; Bilala,b, 2021).

Cryopreservation is one way to ensure the existence of this bird species by conserving its genetics. Sperm cryopreservation is an important technology in avian reproductive biology that allows for the protection and use of genetic resources for commercial and conservation purposes. In contrast, cryopreservation frequently results in a decrease in sperm quality and viability, resulting in a lower fertilization potential and limited reproductive success (Watson et al., 2000; Ali et al., 2022). Therefore, the development of effective cryoprotective techniques to reduce these

adverse effects is critical. To overcome this issue, cryoprotectants, such as butylated hydroxytoluene (BHT), a synthetic derivative of phenol that works as an antioxidant and has antiviral effects, were utilized. BHT has shown promise as a cryoprotectant in various species, including mammals and birds. Reactive oxygen species (ROS) formation, whereas the freezing and thawing procedures involved in sperm cryopreservation pose a danger to the integrity and survival of sperm cells (Afzal et al., 2024; Basharat et al., 2024).

A vital aspect of cryopreservation involves diluting semen with an extender that can maintain both the structural and practical integrity of frozen and melted semen (Sathe, 2021; Aljaser, 2022; Holt, 2000a,b; Rakha et al., 2013; Blanco et al., 2000; Holt, 2000a,b; Blesbois et al., 2008; Rakha et al., 2013; Rakha et al., 2016). The sperm cells of specific species demonstrate distinct biophysical attributes that allow them to interact with a particular extender in highly specialized ways, which undergo transitional changes when subjected to a decrease in temperature (Tasimbaluik, 2021). These interactions can lead to modifications in the structural and functional state of sperm, potentially resulting in negative rather than positive effects (Holt, 2000a,b; Rakha et al., 2016). Aim of this research was to analyze the cryoprotective effects of BHT on sperm parameters in Ring-necked Pheasants during cryopreservation.

MATERIALS AND METHODS

Experimental Design

The study commenced in December 2019 and concluded in August 2020, during which five replicates of semen were examined for each male in the experiment, i.e., semen characteristics. Semen samples were collected once every 24 hours. Semen was collected at 7:00 am for the experiments. For every sample, the sperm cells' volume, concentration, motility, membrane integrity, and acrosome integrity were recorded.

Settings

The research was carried out at the Animal Physiology Laboratory of the Avian Research Center, PMAS-Arid Agriculture University, Rawalpindi, Pakistan.

Sample Size

The sample size was eight mature males (2 years of age). Ring-necked Pheasants were engaged in the study, and they were housed individually in 3.5 × 4 ft cages under standard photoperiod (16HL: 8hD) and temperature settings. Throughout the trial, the birds were fed 100 g per day of commercially accessible poultry-cock breeder feed approved by Islamabad Poultry Feed, as well as freshwater ad libitum.

Sampling Technique

Semen was extracted from the birds (n=8) after four weeks of training (having at least 60% motility and free from fecal contamination) using graduated plastic tubes and belly massage, as described by Burrows and Quinn (1935). To provide clean and uncontaminated samples, birds were trained to collect sperm and promote proper and hygienic semen ejaculation. Using this training method, we successfully collected sufficient sperm for further investigation and handling. We achieved adequate ejaculation of the sperm after a few weeks of training. Each ejaculate was collected and then quickly submerged in a water bath set at 37 °C for analysis. The volume, color, sperm motility, sperm concentration, and sperm morphology of the semen samples were all carefully evaluated. Visual inspection was used to carefully assess the color. The volume of sperm obtained was measured in microliters (ul) using a micropipette. A graduated plastic tube with measurements on it was weighed both before and after semen collection in order to calculate the volume of semen collected. By deducting the tube's starting weight from its weight after the semen was added, the final volume (in liters) was determined.

The initial motility of each sperm sample was determined by combining 10ul of sperm samples with 500ul of phosphate buffer saline (pH 7.2, osmotic pressure 300 mOsm/kg), as described by Zemjanis (1970). The volume of sperm obtained was measured in microliters (ul) using a micropipette. A graduated plastic tube with measurements on it was weighed both before and after semen collection in order to calculate the volume of semen collected. By deducting the tube's starting weight from its weight after the semen was added, the final volume (in liters) was determined. Sperm concentration was determined by combining 1ul of sperm with 200ul of formal citrate solution [1 ml of 37% formaldehyde (CH_2O) to 99 ml of 2.9% (weight/volume) sodium citrate ($Na_3C_6H_5O_7$)] and measured using a Neubauer haemocytometer (counting chamber) (Marienfeld, Germany) under a 400x magnification Phase-contrast microscope (PCM) (Olympus BX20, Japan). By multiplying the entire volume by the concentration, the total number of sperm for each ejaculation was calculated. The total sperm output was multiplied by the corresponding factors to determine the daily sperm output (one unit) and weekly sperm output (seven units) (de-Reviers and Williams, 1981). To ensure the accuracy and dependability of the results, this experiment was conducted three times.

Equipment

Micropipettes, glass slides, slide warmers, a hemocytometer (Neubauer), LN_2 cylinders, a refrigerator, French straws, a water bath, a hot-air oven, and a weighing machine were used in this study.

Sample Selection

Inclusion Criteria

Only samples with motility of more than 75-80% were considered suitable and were included in the analysis for the assessment of semen quality characteristics.

Elimination Criterion

Fresh sperm samples with motility lower than 75% or probable fecal contamination were considered inappropriate and discarded. These samples were not processed or analyzed.

Data Collection Procedure

Extender Preparation and Processing

The researchers wanted to determine how varied dilution strengths and cryoprotectant concentrations affected sperm motility, PMI, viability, and AI. Red Fowl Extender (RFE), developed by modifying the extender suggested by Lake (1978), was used for dilution, which was prepared by combining 100 mL of distilled water with precise concentrations of D-fructose, sodium glutamate ($C_5H_8NO_4Na$), polyvinylpyrrolidone, glycine ($C_2H_5NO_2$), and potassium acetate (CH_3CO_2K). The pH of RFE was 7.0, and the osmotic pressure was 380 mOsm/kg (Purdy et al., 2009). Butylated-hydroxytoluene (BHT) was added in different concentrations. Diluted samples were separated into five groups: A, B, C, D, and E. In cryopreservation investigations, different dilution strengths and cryoprotectant concentrations were used to determine their effects on the quality and preservation of sperm samples. This study attempted to determine the optimal diluent and cryoprotectant concentration for preserving the desired sperm characteristics by diluting the samples at a 1:5 ratio with various extender strengths.

Freezing and Thawing Procedure

Diluted sperm samples ejaculated from eight mature birds were further processed after dilution (1:5) with an extender. They were cooled progressively from 37°C to 20°C and then to 4°C at a regulated rate of -0.275°C per minute for 2h. At 4 °C, 20% glycerol was added after chilling and mixture allowed to equilibrate for 10 min at the same temperature (Purdy et al., 2009). Next, the sperm was filled into 5 mL French straws (IMV, France). These straws carrying the chilled semen were placed at a height of 5 cm in a cold cabinet unit above the level of liquid nitrogen vapor (LN_2). This configuration was maintained for 10 min, allowing the sperm to freeze at a rate of -8.4 °C per minute from 4°C to -80°C. After freezing, the straws were immersed in liquid nitrogen (LN_2) and kept at -196°C for 24 h. This thawing method aided in the evaluation of sperm motility, PMI, viability, and AI.

Sperm Quality Parameters

Sperm Motility

The percentage of sperm moving in a sample is referred to as sperm motility. Minor droplet of previously diluted model was added on a glass slide at 37°C, which served as a platform for measuring sperm motility. A Pasteur pipette is used for this purpose. The slides were then examined under a 400x magnification Phase-contrast microscope (PCM) (Zemjanis, 1970). During this examination, the degree of sperm motility was visually analyzed on a scale varying from 0% to 100%.

Sperm Viability/ Livability

During various stages of cryopreservation, sperm viability, a critical metric for determining sperm quality, was measured using a dual-staining technique with eosin-nigrosin in the lake's glutamate solution.

Sperm Plasma membrane integrity (PMI)

The supravital hypoosmotic swelling test (HOST) has been used at various stages of cryopreservation to determine the integrity of the sperm membrane, which is crucial for sperm survival, fertilization capacity, and osmotic balance maintenance (Santiago-Moreno et al., 2009). A solution was produced for the HOST test by dissolving 1 g sodium citrate ($Na3C_6H_5O_7$) in 100 mL dH_2O.

Sperm Acrosomal Integrity

The acrosome, a critical organelle found in the anterior section of spermatozoa heads, is important for determining acrosomal integrity by Giemsa staining (Jianzhong and Zhang, 2006; Rakha et al., 2015a,b; Rakha et al., 2016). In 35

ml of distilled water, 3 g of Giemsa was combined with 2 ml of Phosphate buffer saline (pH 7.0). The staining process involved smearing a 5ul sample of sperm onto a clean slide and thoroughly aeriated it.

Statistical Analysis of Data

The outcomes are described ±SEM. Fisher's protected LSD test was used to do post hoc comparisons between the means. Moreover, Megastat Version 7.25 Mc-Graw-Hill New Media, New York, for Excel was used to calculate Pearson correlation estimates for semen quality attributes. The experimental results were statistically determined using one-way ANOVA to analyze the effects of various concentrations of butylated hydroxytoluene (BHT) in RFE in different sperm microscopic assays, including sperm motility, viability, PMI, and AI. All treatment groups were compared using the data. Statistical tests were performed at the 95% confidence level.

RESULTS

The effect of various dosages of bovine serum albumin (BSA), a cryoprotectant, on four sperm quality indicators was studied in this study. Motility, PMI, vitality, and AI were the criteria studied. BSA dosages ranging from 0 mg (control) to 0.5, 1, 1.5, and 2 mg were administered to sperm samples. Cryoprotectants were used at various stages and concentrations, including after dilution, chilling, equilibration, and thawing. These numerous treatment conditions allowed for a thorough study of the cryoprotectant's effect on the measured parameters at various phases of the cryopreservation process. To identify the different BHT doses employed in the study, the treatments were labeled T0:0 mM, T1:0.5 mM, T2:1 mM, T3:1.5 mM, and T4:2 mM. These treatment groups were compared to determine how they affected the observed sperm quality metrics, revealing important details regarding the dose-dependent effects of BSA on sperm functionality during cryopreservation.

Sperm Motility

Table 1 shows the effect of butylated hydroxytoluene (BHT) on sperm motility during various stages of cryopreservation in Ring-necked pheasant sperm. In this investigation, BHT treatments included 0.0 mM (as a control), 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM. The data for each parameter are shown in Figure 1.

Post Dilution

The use of 1.0 mM BHT in this phase of cryopreservation had a substantial (88.3±2.8%) effect on sperm motility in the semen extender, outperforming both the control group and other doses ($p > 0.05$). Treatments with 0 mM, 0.5 mM, and 1.5 mM, on the other hand, had identical effects on sperm motility (p-value > 0.05). However, the 2.0 mM treatment had another significant effect, resulting in a motility of 80.0±0.0% (p-value > 0.05), as shown in Table 1. These results show that 1.0 mM BHT had a beneficial effect on sperm motility during cryopreservation at this stage. The remaining concentrations (0.0 mM, 0.5, and 1.5 mM) did not affect motility compared with the control group. Furthermore, the 2.0 mM concentration had a comparable effect on sperm motility, implying that a higher concentration may not always improve sperm motility during cryopreservation.

Post cooling

The study's findings show that 1.0 mM BHT treatment had a considerable (88.3±2.8%) effect on sperm motility in the experimental extender during the post-cooling stage of sperm cryopreservation, likened to the control group and all remaining doses. Doses with 0.0 mM, 0.5 mM, and 2.0 mM, on the other hand, had similar effects on sperm motility (p-value > 0.05). As indicated in Table 1, the 1.5 mM BHT dose had the least effect on sperm motility, with a value of 60.0±0.0% (p-value > 0.05). These data imply that 1.0 mM BHT has a beneficial impact on sperm motility during the cryopreservation post-cooling stage. The remaining values had no impact on the motility of sperm as compared to the control group. Interestingly, among the studied values, 1.5 mM BHT had the least beneficial effect on motility.

Post Equilibration

The 1.0 mM BHT treatment in the semen extender had a considerable (76.6±2.8%) impact on sperm motility, exhibiting a significant difference associated to the control group and all remaining doses. Treatments with 0.5 mM, 1.5 mM, and 2.0 mM, on the other hand, had similar effects on sperm motility (p-value > 0.05). On the other hand, the dose with 0.0 mM BHT had the least impact on sperm motility, with a value of 65.0±5.0% (p-value > 0.05), as shown in Table 1. These findings imply that 1.0 mM BHT had a beneficial effect on sperm motility in Ring-necked Pheasants during the post-equilibration period. In contrast to the control group, motility was unaffected by the residual concentrations. Remarkably, 0.0 mM BHT had the least impact on sperm motility of all the values examined.

Post Thawing

All remaining doses ($p > 0.05$), the study found that the 1.0 mM BHT dose had a considerable (70.0±0.0%) effect on sperm motility in the experimental condition during this phase of sperm cryopreservation. BHT doses of 0.0 mM, 0.5

mM, and 2.0 mM demonstrated alike effects on sperm motility ($p > 0.05$). However, as shown in Table 1, the 1.5 mM BHT therapy had the least effect on sperm motility, with a value of 48.3±2.8% (p-value > 0.05). These results show that the 1.0 mM BHT treatment greatly increased sperm motility during the cryopreservation post-thawing stage. Surprisingly, this increase in motility was consistent across all phases.

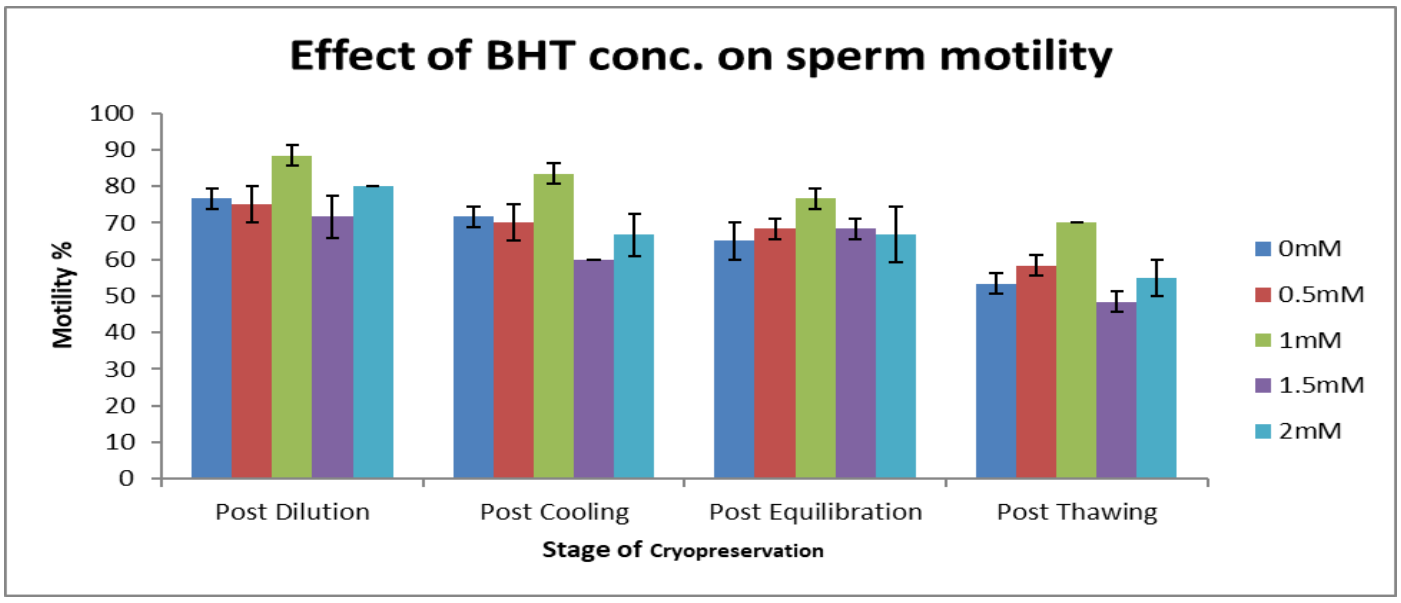

Figure 1. Comparison of BHT concentrations the bird's semen at various stages.

Sperm PMI

BHT's effect on PMI of Ring-necked Pheasant semen at various cryopreservation phases, such as post-dilution, post-cooling, post-equilibration, and post-thawing, is displayed in Table 1. The BHT dosages used in this study were 0.0 mM (control group), 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM each. Data for each parameter is shown in Figure 2.

Post Dilution

When the effects of various concentrations of BHT on the sperm PMI in the semen extender during the post-dilution stage of cryopreservation were compared, it was discovered that the concentration of 1.0 mM had a considerable effect (90.3±1.5%) when compared with the control group and all other treatments ($p > 0.05$). While 1.5 mM had the least effect (74.6±5.7%) on the sperm PMI (p-value > 0.05), doses of 0.0 mM, 0.5 mM, and 2.0 mM had comparable effects (p-value > 0.05), as indicated in Table 1.

Post-cooling

In comparison to a control group (0 mM) and all other dosages during this stage of sperm cryopreservation, the use of BHT at a dose of 1.0 mM had a significant impact (88.0±2.0%) on the sperm PMI in the experimental extension. Sperm plasma membrane integrity was affected in the same way by 0 mM and 0.5 mM concentrations, with values of 83.0±3.0% and 82.6±4.0%, respectively ($p > 0.05$). Additionally, Table 1 indicates that the effects of 1.5 mM and 2.0 mM doses on the integrity of the sperm plasma membrane were comparable (p-value > 0.05).

Post Equilibration

The data on the effect of BHT on sperm PMI of Ring-necked pheasant at this stage revealed that BHT at 1.0 mM dose had a significant effect (i.e., 86.3±1.5%) in semen extender on sperm PMI ($p > 0.05$) as compared to the control group (0 mM) and all other doses. The concentrations of 0.5 mM, 1.5 mM, and 2.0 mM have essentially identical effects on sperm PMI ($p > 0.05$), but there was another substantial effect on sperm PMI at 0 mM, i.e., 82.6±2.0% (p-value > 0.05).

Post-thawing

In comparison to the control group (0.0 mM) and all other doses, the results of the post-thawing stage of sperm cryopreservation showed that BHT at a 1.0 mM dose had a significant effect (i.e., 78.6±3.2%) in the experimental extender on sperm PMI ($p > 0.05$). Sperm PMI was about the same at 0.5 mM and 2.0 mM doses (70.03.6%) and 69.31±2.8%), respectively ($p > 0.05$). There is a comparable effect on sperm PMI at 0.0 mM and 1.5 mM concentrations, specifically 65.6±7.5% and 66.0±5.2%) (p-value > 0.05).

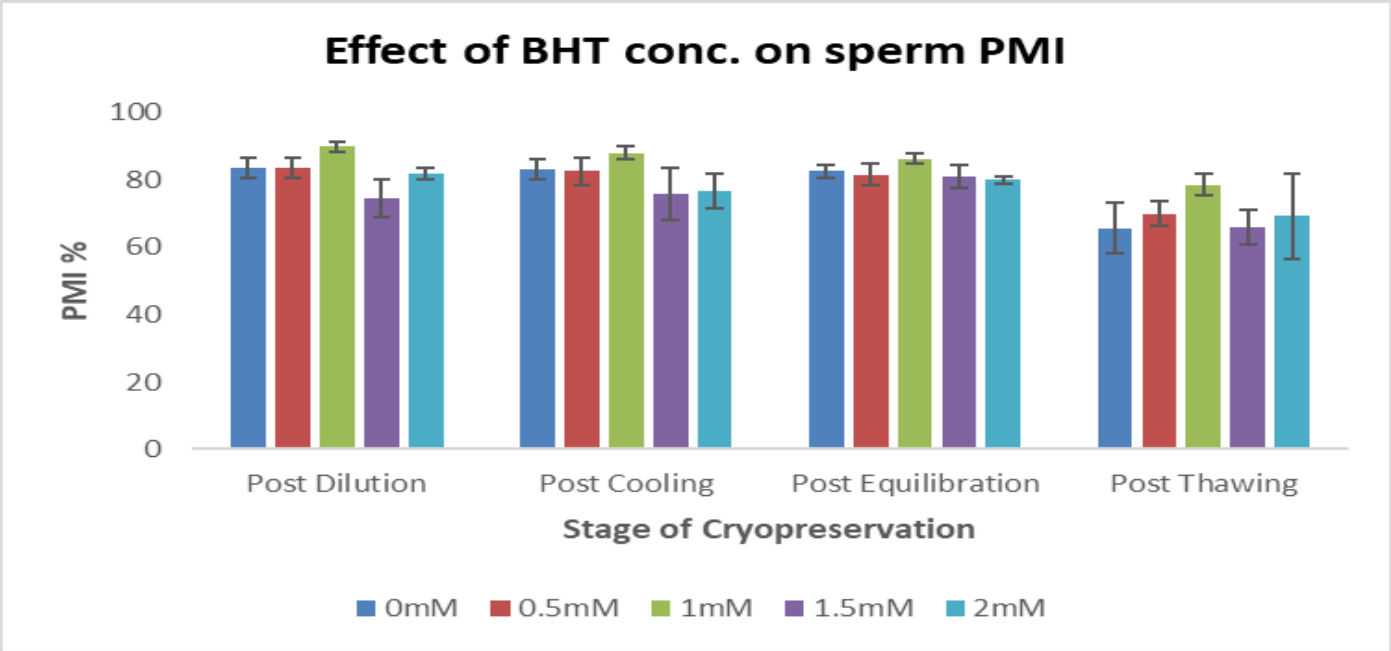

Figure 2. Comparison of BHT concentrations (0.0.0 mM, 1.0 mM, 1.5mM, and 2.0 mM) on sperm plasma membrane integrity (%) of Ring-necked Pheasant semen at various stages of cryopreservation along the x-axis. The erroe bars differ significantly ($p<0.05$) at a given time.

All phases of sperm plasma membrane integrity post-dilution, post-cooling, post-equilibration, and post-thawing were significantly impacted by the 1.0 mM treatment.

Sperm Livability

Butylated hydroxytoluene's impact on the microscopic characteristics of Ring-necked Pheasant semen and its viability during different phases of cryopreservation, including post-dilution, post-cooling, post-equilibration, and post-thawing, is shown in Table 1. In this investigation, BHT concentrations of 0.0 mM (control group), 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM were used. Figure 3 displays the data for each of the parameters.

Post Dilution

During the post-dilution phase of cryopreservation, BHT at a 1.0 mM dose significantly affected sperm viability (i.e., 88.3±2.3%) in semen extender when compared to the control group and all other doses ($p > 0.05$). The 1.5 mM treatment had the lowest effect, at 74.34.6% ($p > 0.05$), whereas the 0.0 mM and 0.5 mM treatments had almost the same effects on sperm viability, at 80.0±3.4%) and 80.6±3.7%), respectively. Additionally, sperm viability was significantly impacted by BHT at a dose of 2.0 mM (82.0±2.6 5%; p-value > 0.05).

Post-cooling

BHT at a 1.0 mM dose had a significant impact (i.e., 85.3±2.0%) on sperm vitality in the experimental extender ($p > 0.05$) as compared to the control group (0.0 mM) and all other doses, according to data on the post-cooling stage of sperm cryopreservation. Sperm viability at 0.0 mM and 0.5 mM was almost the same, at 75.6±1.5% and 74.6±2.5%, respectively ($p > 0.05$). Additionally, sperm viability was least affected by the 1.5 mM and 2 mM doses (67.3±2.5% and 70.3±8.6%, respectively) ($p > 0.05$).

Post Equilibration

Following equilibration, the impact of BHT on sperm viability in Ring-necked Pheasants was investigated. According to the data, sperm viability in the semen extender was significantly impacted by BHT (1.0 mM) compared to the control group (0.0 mM) and all other doses ($p > 0.05$). However, there was no significant difference in the effects of 0.5 mM, 1.5 mM, and 2.0 mM BHT on sperm viability (p-value > 0.05). Of the concentrations examined, Table 1 demonstrates that the dose containing 0.0 mM BHT had the least impact on sperm viability (72.6±2.5%) ($p > 0.05$).

Post-thawing

During this stage, BHT at 1.0 mM was shown to have a significant (72.6±2.5%) impact on sperm viability in the experimental extender than a control group (0.0 mM) and all remaining doses (p-value > 0.05). The 1.5 mM and 2.0

mM BHT treatments had identical effects on the viability of sperm (66.6±7.0% and 66.6±12.6%, respectively) (p-value > 0.05). Table 1 shows that the dose with 0.0 mM BHT had the least effect on sperm viability, with a value of 68.3±10.0% (p-value > 0.05). Notably, 1.0 mM BHT administration remarkably altered all phases of sperm.

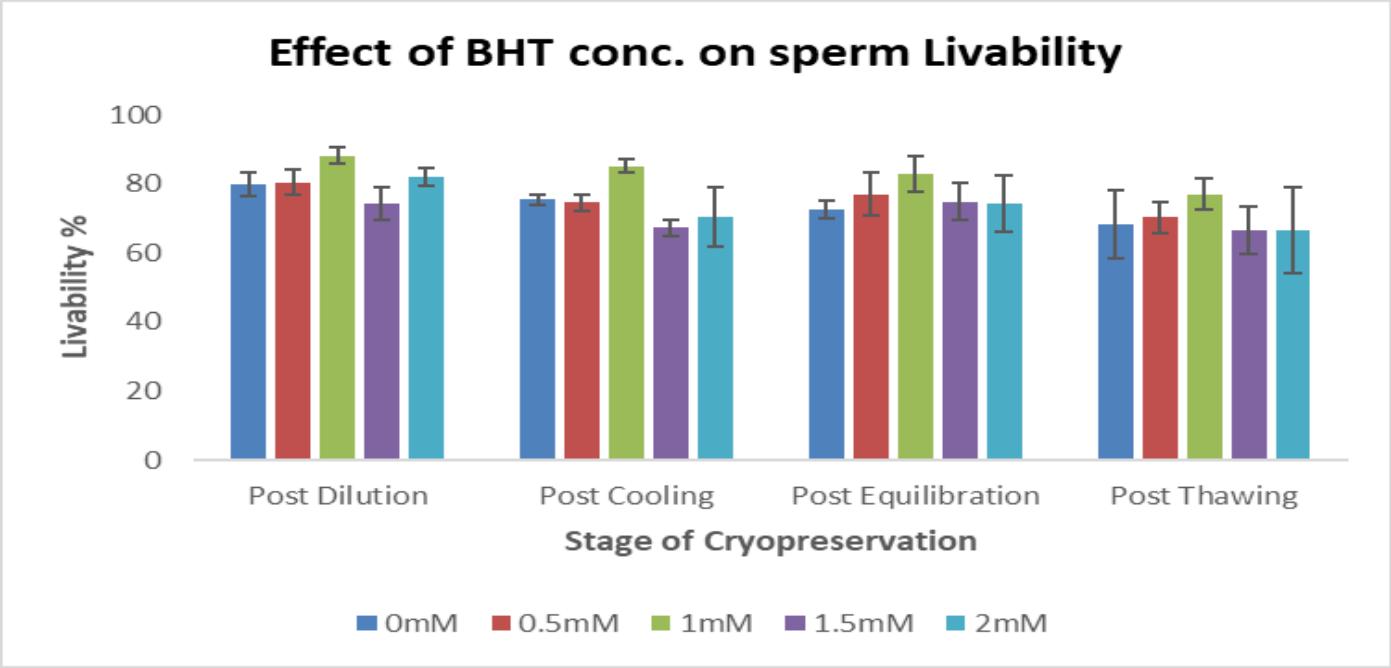

Figure 3. Comparison of BHT concentrations (0.0.0 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM) on sperm Livability.

Sperm Acrosome Integrity

Table 1 shows the effect of BHT on the AI of Ring-necked Pheasant semen at different stages of cryopreservation, including post-dilution, post-cooling, post-equilibration, and post-thawing. The following BHT concentrations were used in the study: 0.0 mM (as a control), and the remaining all groups Figure 4.

Post Dilution

The application of 1.0 mM BHT during the post-dilution phase of cryopreservation substantially influenced (86.6±2.8%) sperm acrosomal integrity in the semen extender than in the control group and all remaining doses (p > 0.05). Concentrations of 0.0 mM and 2.0 mM had identical impacts on the acrosomal integrity of sperm (p-value > 0.05) (80.3±3.5% and 81.0±2.6%, respectively). Similarly, the 0.0 mM and 1.5 mM doses had equivalent effects on sperm acrosomal integrity (77.6±2.0% and 76.0±5.2%, respectively) (p > 0.05).

Post Cooling

The use of a 1.0 mM BHT therapy during the post-cooling stage of sperm cryopreservation had a substantial impact on the AI of sperm (85.3±2.0%) than a control group (0.0 mM) and all remaining doses (p-value > 0.05). Doses of 0.0 mM, 0.5 mM, and 2.0 mM had importantly equivalent effects on the acrosomal integrity of sperm, yielding values of 76.0±4.0%, 76.6±2.5%, and 77.0±5.0% (p-value > 0.05). Furthermore, the 1.5 mM dose had the least effect on sperm acrosomal integrity, measuring 71.0±6.0% (p-value > 0.05), as shown in Table 1.

Post Equilibration

The effect of BHT on the acrosomal integrity of sperm in Ring-necked Pheasants was also studied. A 1.0 mM BHT treatment significantly improved sperm acrosomal integrity in the semen extender, yielding a value of 82.6±3.0% (mean standard deviation). This impact differed significantly from both groups (0.0 mM as a control) and all remaining doses (p-value > 0.05). Doses of 0.0 mM, 0.5 mM, 1.5 mM, and 2.0 mM, on the other hand, had nearly equal effects on sperm acrosomal integrity, with no significant changes found (p-value > 0.05).

Post Thawing

In the experimental extension, the use of a 1.0 mM BHT treatment significantly reduced sperm acrosomal integrity (79.0±2.6%) throughout the post-thawing stage of sperm cryopreservation as compared to both the control group (0.0 mM) and all remaining doses (p > 0.05). The effects of 0.0 mM and 0.5 mM on the sperm acrosomal integrity were

similar, with values of 70.0±9.1% and 72.6±7.5%, correspondingly (p-value > 0.05). Furthermore, as demonstrated in Table 1, doses of 1.5 mM and 2.0 mM had equivalent effects on sperm AI, with the values of 65.6±8.5% and 66.6±11.3%, accordingly (p-value > 0.05). The application of 1.0 mM significantly improved acrosomal integrity during the post-thawing stage of cryopreservation compared to the control group and other doses. However, doses of 0.0 mM, 0.5 mM, 1.5 mM, and 2.0 mM had no significant effects on sperm acrosomal integrity.

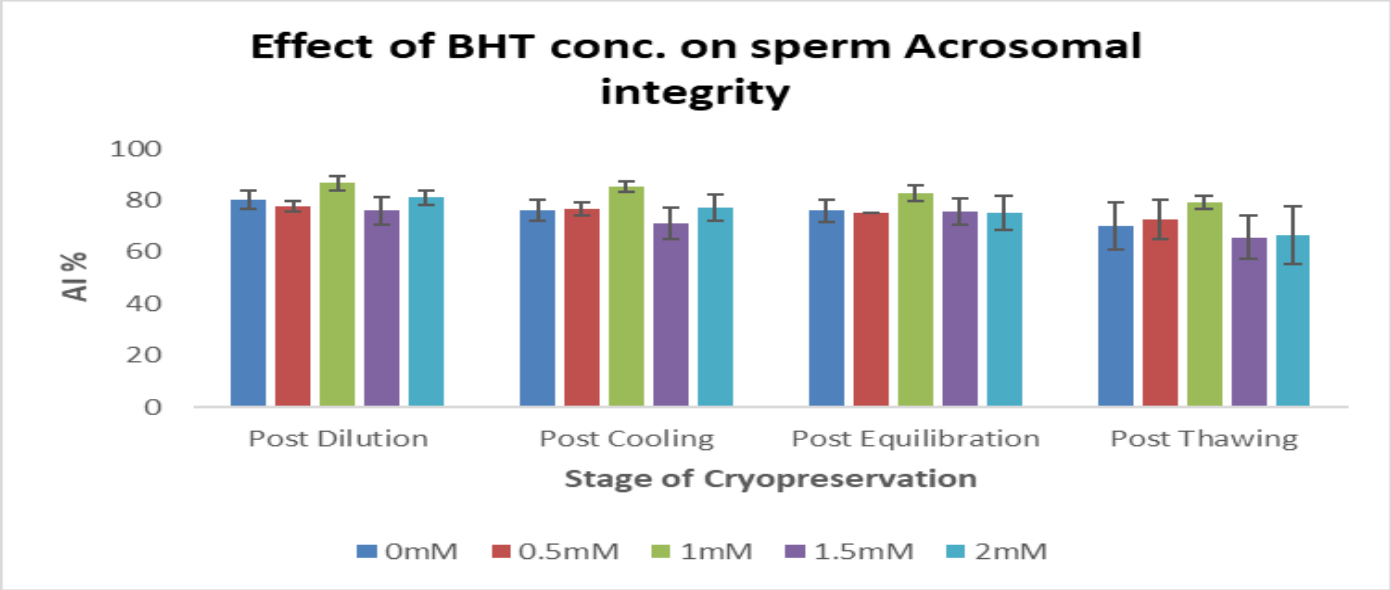

Figure 4. Comparison of BHT concentrations (0.0.0 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM) on sperm AI.

Table 1 lists the characteristics of Ring-necked Pheasant sperm at various phases of cryopreservation, including sperm motility, sperm PMI, viability, and AI. It is important to note that the data were assessed using one-way ANOVA. The mean and standard deviation were calculated for every parameter.

Table 1. Ring-necked Pheasant sperm characteristics, including mean values and standard errors of the mean (SEM) for various sperm parameters. The data is based on an 8-sample size (n=8)

Sperm Quality Parameters	0.1mM	0.5mM	1mM	1.5mM	2mM
Post dilution	71.6±2.8	70.0±5.0	88.3±2.8	71.6±5.7	80.0±0.0
Post cooling	71.6±2.8	70.0±5.0	88.3±2.8	60.0±0.0	66.6±5.7
Post equilibration	65.0±5.0	68.3±2.8	76.6±2.8	68.3±2.8	66.6±7.6
Post thaw	53.3±2.8	58.3±2.8	70.0±0.0	48.3±2.8	55.0±5.0
Post dilution	83.6±3.2	83.6±3.2	90.3±1.5	74.6±5.7	82±1.7
Plasma Membrane Integrity					
Post cooling	83.0±3.0	82.6±4.0	88.0±2.0	76.0±7.8	76.6±5.1
Post equilibration	82.6±2.0	81.6±3.2	86.3±1.5	81.0±3.6	80.0±1.0
Post thaw	65.6±7.5	70.0±3.6	78.6±3.2	66.0±5.2	69.3±12.8
Livability					
Post dilution	80.0±3.4	80.6±3.7	88.3±2.3	74.3±4.6	82.0±2.6
Post cooling	75.6±1.5	74.6±2.5	85.3±2.0	67.3±2.5	70.3±8.6
Post equilibration	72.6±2.5	77.0±6.2	83.0±5.0	75.0±5.2	74.3±8.1
Post thaw	68.3±10.0	70.3±4.7	77.0±4.5	66.6±7.0	66.6±12.6
Acrosome Integrity					
Post cooling	76.0±4.0	76.6±2.5	85.3±2.0	71.0±6.0	77.0±5.0
Post equilibration	7.0±4.3	75.0±0.0	82.6±3.0	75.6±4.9	75.0±6.5
Post thaw	70.0±9.1	72.6±7.5	79.0±2.6	65.6±8.5	66.6±11.3

DISCUSSION

Reactive oxygen species (ROS), which are produced in large quantities by sperm cells, are essential for intercellular signaling mechanisms that result in sperm capacitation, acrosome response, hyperactivation, and fusion with the oocyte (Baumber et al., 2003). The generation of ROS can be harmful even if it might be beneficial in certain situations (Nordberg and Amer, 2001). Oxidative stress, which occurs when spermatozoa have too many ROS, can cause the plasma membrane to break down, which reduces sperm motility, metabolic activity, lifespan, and viability (Ball et al., 2001). Damaged spermatozoa, infiltrating leukocytes, or spermatozoa with remaining cytoplasm are usually the causes of excessive ROS production (Pagl et al., 2006).

Because of the large levels of phospholipid and unsaturated fatty acids in their membranes, mammalian sperm are especially vulnerable to lipid peroxidation (Aitken et al., 1993). Lipid peroxidation, which is caused by ROS, might result in decreased semen quality. But antioxidants found in seminal plasma guard against this process (Dawra and Sharma, 1985; Strzezek et al., 1999). For long-term storage, this degree of protection might not be enough, though. Increased oxidative stress occurs during reproductive procedures, or the antioxidants in the seminal plasma are eliminated during the cryopreservation preparation of the sperm. Additionally, there is proof that the freeze-thaw cycle itself may cause more ROS to be formed in the semen, which would further harm the sperm (Chatterjee et al., 2001). Thus, the antioxidant system of the semen can be controlled by adding exogenous antioxidants. There are both positive and negative effects on the survival of spermatozoa from various animal species when BHT is added to preserved semen. The addition of BHT to the cooling and freezing media improved the survival of cold-stressed cattle spermatozoa (Graham and Hammerstedt, 1992), cryopreserved cattle spermatozoa (Killian et al., 1989), buffalo spermatozoa (Ijaz et al., 2009), dog sperm (Neagu et al., 2010), and chilled turkey tom sperm (Donoghue and Donoghue, 1997).

Ball et al. (2001) found that BHT supplementation had a negative impact on cooled stallion spermatozoa. These findings suggest that there are still major obstacles to overcome in terms of species differences and technical proficiency when utilizing BHT to cryopreserve semen. BHT's strong antioxidant qualities have been linked to its capacity to increase sperm motility. Oxidative stress brought on by cryopreservation can result in lipid peroxidation and the generation of reactive oxygen species (ROS), both of which can impair sperm motility. By neutralizing ROS and lowering oxidative damage in sperm cells, BHT improves sperm motility and acts as an antioxidant. Furthermore, by halting lipid peroxidation and preserving membrane structure and function, BHT has been demonstrated to safeguard sperm viability and plasma membrane integrity.

This is crucial because sperm plasma membrane damage from cryopreservation can reduce their viability. Additionally, BHT enhanced the acrosomal integrity of the sperm. Because it allows sperm to enter eggs, the acrosome is essential for fertilization. The fertilization process may be jeopardized by cryopreservation-induced acrosome injury. BHT guarantees sperm cell function during fertilization by preserving the acrosomal integrity. These results clearly showed the protective impact of antioxidant supplementation on sperm, supporting the findings of Roca et al. (2004), Shoae and Zamiri (2008), Ijaz et al. (2009), Neagu et al. (2010), and Donoghue and Donoghue (1997).

The spermatozoa of dogs, boars, cattle, buffalo, and turkeys were cryopreserved by these researchers using BHT. The protective benefits of BHT have been explained by two potential mechanisms. First, it is believed that BHT is integrated into spermatozoa's plasma membrane, where it improves membrane fluidity and shields the sperm from harm (Aitken and Clarkson, 1987). Second, BHT is thought to give sperm further protection by converting lipid peroxyl radicals into hydroperoxides, which stops their harmful activity (Aitken and Clarkson, 1987).

This study is the first to use BHT as a cryoprotectant to gather and assess microscopic data and Ring-necked Pheasant sperm characteristics. However, there were no discernible variations between the eight experimental birds in terms of microscopic measurements such as sperm, motility, PMI, viability, and AI ($p > 0.05$). This constant conclusion may be explained by the ages of the birds, their consistent feeds, and the equal cage management conditions. All phases of sperm cryopreservation, including sperm motility, PMI, viability, and AI, were significantly ($p > 0.05$) impacted by 1.0 mM BHT. When RFE (red flower extender) was used with the BHT extender, this result was seen. After cryopreservation, BHT at 1.0 mM was found to be more effective than the control group at improving sperm quality.

These results corroborate earlier research that suggested BHT had cryoprotective benefits for birds. Therefore, there is potential for employing BHT 1.0 mM to enhance sperm quality during cryopreservation in Ring-necked Pheasants. Previous studies have shown that spermatozoa can be harmed by high BHT concentrations. However, several normal physiological processes including sperm hyperactivation, capacitation, and acrosome response are linked to the controlled production of reactive oxygen species (ROS) in spermatozoa (Aitken, 1995; Shahin et al., 2024). The

extender can counteract the oxidative stress brought on by excessive ROS creation by adding excessive levels of antioxidant chemicals to the semen, but it can also interfere with normal sperm functions linked to ROS. In order to preserve the natural equilibrium between ROS formation and scavenging activity, it is imperative to select an adequate antioxidant concentration. Similar findings have been documented in swine sperm cells (Pursel, 1979; Iftikhar et al., 2024; Sattar et al., 2024; Bamba and Cran, 1992). They discovered that better outcomes were obtained with BHT values between 0.05 and 2 mM. However, because it loses its protective action on Ring-necked Pheasant spermatozoa, the greatest concentration assessed in this study (1.0 mM) was not higher than 2.0 mM BHT. When quantities of BHT approached 2 mM, its protective effect against cold stress on spermatozoa was no longer present (Bamba and Cran, 1992). BHT appears to have both positive and negative effects on post-thawed sperm when added to semen extenders.

Our findings showed that BHT can successfully reduce the negative effects of cryopreservation on sperm parameters in Ring-necked Pheasants. These findings are important for developing optimum cryopreservation techniques for this species as well as for the conservation and management of other vulnerable avian species. Recognizing the limitations of this study is critical. First, further research into the appropriate concentration and the duration of BHT treatment is needed. Different concentrations of BHT may provide different benefits, and further research is required to determine the optimal dosage. Second, the long-term effects of BHT on fertility rate and embryonic development are unknown. Further research should be conducted to evaluate the reproductive outcomes of cryopreserved semen treated with BHT after artificial insemination.

CONCLUSIONS

The experimental results show that using 1.0 mM BHT has a considerable ($p > 0.05$) impact on various quality assays related to sperm quality throughout all different stages of cryopreservation, including post-dilution, post-cooling, post-equilibration, and post-thawing. Sperm motility, PMI, viability, and AI are among these criteria. BHT's antioxidant properties of BHT contribute to its beneficial effects. BHT functions as an antioxidant, assisting in the prevention of oxidative stress and the reduction of damage caused by reactive oxygen species (ROS) and lipid peroxidation. During cryopreservation, BHT functions as a ROS scavenger, maintaining sperm quality and increasing reproductive capacity. The findings of this study have crucial implications for improving cryopreservation techniques in Ring-necked Pheasants, and possibly other bird species. Researchers and conservationists can improve the long-term survival and reproductive success of these species by integrating BHT with cryopreservation methods. This represents a promising way to preserve the genetic variety of bird populations while also assisting in conservation efforts.

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AUTHOR CONTRIBUTIONS

The experiments were mainly carried out by A.I and A.B. while A.I wrote the manuscript. B.A.R. and S.A. supervised the research and A.B collected the data and analyzed it. All authors have read and approved the manuscript.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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