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VITAMIN C SUPPLEMENTATION IN EGG YOLK CITRATE EXTENDER OPTIMIZES BANGKOK ROOSTER SEMEN QUALITY DURING COLD STORAGE

Suplementasi Vitamin C dalam Pengencer Sitrat Kuning Telur untuk Mengoptimalkan Kualitas Semen Ayam Bangkok Selama Penyimpanan Dingin

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ABSTRACT
This study aimed to evaluate the effect of different vitamin C concentrations (0, 0.1, 0.2, and 0.3 g/100 mL) in egg yolk citrate extender and storage time (0, 24, 48, 72, and 96 hours) on the quality of Bangkok rooster semen stored at 5°C. Using a split-plot-in-time design with four replications, semen quality was assessed for motility, viability, abnormality, intact plasma membrane (IPM), and longevity. Results showed a significant interaction (P<0.01) between vitamin C concentration and storage time on motility, and significant effects (P<0.05) on viability, abnormality, and IPM. The 0.2 g vitamin C dose preserved >40% motility and >50% viability for 96 hours and extended sperm longevity up to 11.25±0.95 days. These findings suggest that 0.2 g/100 mL vitamin C is the optimal concentration for preserving rooster semen quality during short-term cold storage.

Keywords: *Bangkok rooster, Extender, Liquid semen, Sperm quality, Vitamin C*

ABSTRAK
Penelitian ini bertujuan untuk mengevaluasi pengaruh penambahan vitamin C dengan dosis berbeda (0, 0,1, 0,2, dan 0,3 g/100 mL) dalam pengencer sitrat kuning telur serta waktu penyimpanan (0, 24, 48, 72, dan 96 jam) terhadap kualitas semen ayam Bangkok yang disimpan pada suhu 5°C. Penelitian menggunakan rancangan petak terbagi dalam waktu dengan empat ulangan. Parameter yang diamati meliputi motilitas, viabilitas, abnormalitas, integritas membran plasma (IMP), dan longivitas. Hasil analisis ragam menunjukkan adanya interaksi yang sangat nyata (P<0,01) antara dosis vitamin C dan waktu penyimpanan terhadap motilitas, serta pengaruh yang nyata (P<0,05) terhadap viabilitas, abnormalitas, dan IMP. Perlakuan dengan dosis vitamin C 0,2 g mampu mempertahankan motilitas >40% dan viabilitas >50% selama 96 jam penyimpanan, serta menghasilkan longivitas sperma tertinggi hingga 11,25±0,95 hari. Penambahan vitamin C dosis 0,2 g/100 mL terbukti sebagai konsentrasi optimal untuk mempertahankan kualitas semen ayam Bangkok selama penyimpanan dingin jangka pendek.

Kata Kunci: *Ayam Bangkok, pengencer SKT, Kualitas spermatozoa, Semen cair, Vitamin C*

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INTRODUCTION

Artificial insemination (AI) has become a cornerstone of genetic improvement and reproductive management in poultry, enabling wider dissemination of superior genetic traits while minimizing disease transmission risks. The success of AI programs largely depends on the ability to preserve sperm quality during storage, which requires optimized extenders and protective additives (Surai and Wishart 1996; Sharma et al. 2024). While frozen semen is widely used in mammals, liquid storage remains the preferred method for poultry due to higher post-thaw damage associated with cryopreservation in avian spermatozoa (Ramírez-Reveco et al. 2016; Al-Bulushi et al. 2019). However, even under chilled conditions, rooster sperm rapidly deteriorates in terms of motility, viability, and membrane integrity due to metabolic activity and oxidative stress (Kadili et al. 2020; Guo et al. 2023).

Oxidative stress, driven by excessive production of reactive oxygen species (ROS), is a major factor contributing to sperm quality decline during semen storage. It disrupts plasma membrane integrity, DNA stability, and enzymatic function, ultimately impairing sperm motility and fertilizing capacity (Aitken and Baker 2002; Silvestre et al. 2021). To mitigate these effects, antioxidants are frequently added to semen extenders. Among them, vitamin C (ascorbic acid) has gained prominence for its water-soluble antioxidant properties, which include scavenging free radicals and reducing lipid peroxidation in sperm membranes (Amini et al. 2015; Partyka and Niżański 2021).

In poultry, several studies have shown that vitamin C supplementation in semen extenders enhances post-thaw motility, viability, and fertilizing potential (Yahaq et al. 2019; Akhter et al. 2023). Notably, Amini et al. (2015) demonstrated that vitamin C at concentrations of 2.5–4.5 mg/mL significantly improved sperm quality by reducing malondialdehyde (MDA), a key marker of oxidative damage. However, findings also indicate that vitamin C's protective effects are dose-dependent, with higher concentrations potentially exerting pro-oxidant effects and negatively altering pH, leading to impaired sperm viability (Gangwar et al. 2015; Echekwu et al. 2021; Attia et al. 2020).

Egg yolk citrate extender has been widely used in poultry semen preservation due to its buffering capacity and membrane-stabilizing lipoproteins, particularly low-density lipoproteins (LDL), which help maintain sperm membrane integrity during cold storage (Sarder et al. 2013; Ford 2003). The synergistic use of vitamin C and egg yolk citrate has been shown to enhance sperm motility and viability, especially in rooster semen which is highly susceptible to oxidative damage due to its high polyunsaturated fatty acid (PUFA) content (Șipotenu et al. 2023; Diba et al. 2023).

Despite these findings, limited studies have evaluated the combined effect of different vitamin C concentrations in egg yolk citrate extender on semen quality in Bangkok roosters, a breed valued for its endurance and cultural significance in Southeast Asia. Moreover, research is needed to determine the optimal dosage of vitamin C that balances its antioxidant potential without inducing oxidative or pH-related stress.

In the present study, vitamin C was supplemented into egg yolk citrate extender at doses of 0, 0.1, 0.2, and 0.3 g per 100 mL. These concentrations were selected based on prior research indicating that optimal antioxidant effects are achieved within a narrow concentration range. Doses below 0.1 g/100 mL may be insufficient to counteract ROS, while doses above 0.3 g/100 mL risk inducing pro-oxidant activity that can paradoxically damage spermatozoa (Echekwu et al. 2021; Attia et al. 2020). The 0.2 g/100 mL dosage was specifically chosen as a mid-range value, in line with findings by Akhter et al. (2023) and Amini et al. (2015), who reported that moderate concentrations of vitamin C significantly improved sperm motility and viability during chilled storage in poultry and other livestock species. By including lower and higher doses alongside a control, this study aimed to determine the threshold at which vitamin C transitions from beneficial to detrimental for Bangkok rooster semen under chilled storage.

Therefore, the aim of this study was to evaluate the interaction between vitamin C doses in egg yolk citrate extender and stor-

age duration at 5°C on the quality of Bangkok rooster semen, focusing on key parameters such as motility, viability, abnormality, plasma membrane integrity, and longevity.

MATERIALS AND METHODS

Location and Experimental Period

The experiment was conducted from January to March 2025 at the UPT Farm and Animal Biotechnology Laboratory, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia.

Animals and Semen Collection

Three sexually mature Bangkok roosters, each two years old and averaging 3 kg body weight, were used as semen donors. The birds were maintained according to standard management practices. Semen was collected using the dorsoabdominal massage technique between 07:00 and 09:00 a.m. after 12 h of fasting. The cloacal area was cleaned, and the ejaculate was collected in sterile 1.5 mL microtubes. Ejaculates with ≥70% motility and <20% abnormalities were pooled and used for subsequent procedures.

Extender Preparation and Treatments

The citrate buffer was prepared by dissolving 1.25 g of fructose and 2.32 g of sodium citrate in 100 mL of distilled water, as shown in Table 1. To produce the extender, 80 mL of citrate buffer was combined with 20% egg yolk (v/v), followed by the addition of 1000 IU penicillin and 1 mg streptomycin. Vitamin C was then added at concentrations of 0, 0.1, 0.2, and 0.3 g per 100 mL to formulate four treatment groups (SKT, SKT-C 0.1, SKT-C 0.2, and SKT-C 0.3). Each formulation was thoroughly homogenised before being used to dilute the pooled semen.

Tabel 1. Evaluation of fresh semen quality parameters in Bangkok roosters

Evaluation Type	Parameter	Mean ± SD
Macroscopic	Volume (mL)	0.32 ± 0.13
	Color	Cream
	Consistency	Viscous
	pH	7.16 ± 0.37
Microscopic	Mass Movement	(++ - +++)
	Motility (%)	86.66 ± 4.71
	Viability (%)	92.91 ± 3.66
	Abnormality (%)	8.91 ± 1.60
	Concentration (million/ejaculate)	2247.66 ± 261.56
	Intact Plasma Membrane (%)	91.41 ± 3.57

Semen Dilution

Semen was collected from three healthy, sexually mature Bangkok roosters using the dorsal-abdominal massage technique. Only ejaculates with total motility above 70% and morphological abnormalities below 20% were selected. These quality thresholds are widely used in poultry semen evaluation to ensure the use of functionally competent ejaculates for further processing (Akhter et al. 2023; Yahaq et al. 2019). Qualified semen samples were pooled into a sterile 15 mL tube to eliminate individual variation. Sperm concentration was assessed using a hemocytometer after dilution in 3% NaCl, and calculated as follows:

$$Sperm\ concentration\ (10^6) = average\ cell\ count\ in\ five\ squares \times 25 \times dilution\ factor$$

The semen was then adjusted to a final concentration of 200 million spermatozoa per mL, based on recommendations for optimal insemination dosing in poultry (Iorio et al. 2020; Thélie et al. 2019). Research has shown that this concentration is effective in maintaining fertility rates while allowing efficient use of extenders. Each 1 mL of semen was diluted with egg yolk citrate extender supplemented with varying concentrations of vitamin C. The experimental groups were P0 (citrate extender without vitamin C (control)), P1 (citrate extender + 0.1

g vitamin C/100 mL), P2 (citrate extender + 0.2 g vitamin C/100 mL), P3 (citrate extender + 0.3 g vitamin C/100 mL).

Dilution was performed gently using a micropipette along the inner wall of the tube to minimize mechanical damage to sperm cells. Aliquots of 1.5 mL were distributed into microtubes and stored at 5°C. Evaluations were conducted at 0, 24, 48, 72, and 96 h, covering parameters of motility, viability, abnormality, intact plasma membrane (IPM), and longevity, with four replications per treatment group.

Semen Evaluation

Fresh semen was evaluated immediately after collection to determine eligibility for processing. The evaluation included both macroscopic and microscopic assessments of the samples. The macroscopic parameters included volume (recorded from the collection tube), color (milky white, cream, or yellow), and consistency (watery, medium, or thick). The pH of fresh semen was measured immediately after collection using pH indicator strips (range 6.0–8.0) with an accuracy of ±0.1 pH units. A small drop of semen was placed directly onto the pH paper, and the resulting color change was compared against the reference scale provided by the manufacturer. Measurements were performed in duplicate to ensure consistency, and the average was recorded for further analysis. Microscopic evaluations included mass movement, progressive motility, concentration, viability, abnormality, and intact plasma membranes (IPM).

Mass movement was assessed by placing 2 µL of semen on a glass slide and observing it under a microscope at 100× magnification. The intensity of wave motion was scored from “excellent” (+++), indicating dense and rapid waves, to “poor” (N/0), indicating no visible movement of the waves. Progressive motility was evaluated at 400× magnification by mixing one drop of semen with four drops of physiological saline on a slide and covering it with a coverslip. Motility scores were assigned according to Arifiantini (2012), ranging from 0% (no movement) to 90–100% (strong, progressive movement with rapid wave formation).

Sperm concentration was determined by diluting 2 µL of semen in 998 µL of 3% NaCl. A 10 µL aliquot was placed into a Neubauer hemocytometer chamber and observed under a microscope at 400× magnification. The number of spermatozoa in five counting squares was multiplied by 25×10^6 to obtain the final concentration (Arifiantini 2012).

Sperm viability was evaluated using eosin staining. A small drop of semen (approximately 10 µL) was mixed with 8–10 drops of 2% eosin Y solution, allowed to react for 30 seconds, then smeared onto a clean glass slide. The slide was air-dried at 37°C and observed under a microscope at 400× magnification. Viable spermatozoa remained unstained (transparent heads), while non-viable spermatozoa showed red or purple-stained heads due to membrane damage allowing dye penetration. A minimum of 200 cells was counted across 10 random fields, The percentage of viable sperm was calculated as the number of live sperm divided by the total number of sperm counted multiplied by 100% (Arifiantini 2012).

Abnormalities were evaluated on unstained smears by observing at least 200 sperm cells across 10 fields. The percentage of abnormal sperms was calculated based on the total number of spermatozoa.

IPM integrity was assessed using the hypoosmotic swelling test (HOST). Two microliters of fresh semen were added to 998 µL of HOST solution and incubated at 37°C for 45 min. A drop of the mixture was placed on a pre-warmed slide, covered with a coverslip, and examined at 400× magnification. Spermatozoa with coiled tails were considered to have intact membranes.

After dilution with the respective extenders, the semen samples (liquid semen) were stored at 5°C. Observations were conducted every 24 h, starting from 0 h to 96 h. The evaluated parameters included progressive motility, viability, abnormalities, and intact plasma membranes (IPM). Additionally, longevity was assessed by monitoring sperm motility daily at room temperature (24–28°C) until all spermatozoa had completely lost motility. Longevity was defined as the total duration from the beginning of storage to the complete cessation of motility.

Data Analysis

The experiment was arranged using a split-plot-in-time design with four replications. The main plot factor was the type of extender—consisting of four treatments: 0 (control), 0.1, 0.2, and 0.3 g vitamin C per 100 mL—while the subplot factor was storage duration (0, 24, 48, 72, and 96 hours). Treatments were randomly assigned to experimental units, and semen from each treatment group was evaluated at each time point. All quantitative data were analyzed using two-way Analysis of Variance (ANOVA) to assess the effects of extender type, storage duration, and their interaction on semen quality parameters. Duncan's Multiple Range Test (DMRT) was applied as a post hoc test when the ANOVA revealed statistically significant differences ($P < 0.05$) or highly significant differences ($P < 0.01$). Statistical analyses were performed using IBM SPSS Statistics version 22. All data were checked for normality and homogeneity of variance prior to analysis.

RESULTS AND DISCUSSION

Fresh Semen Quality of Bangkok Roosters

The fresh semen quality of Bangkok roosters observed in this study was characterized by satisfactory macroscopic and microscopic parameters (Table 1). The average semen volume was 0.32 ± 0.13 mL, with a thick (viscous) consistency and creamy color. The semen exhibited a slightly alkaline pH of 7.16 ± 0.37, which falls within the physiological range (6.0–8.0) previously reported in roosters and is essential for maintaining sperm viability during storage (Udrayana et al. 2023; Sutiyono et al. 2021).

Microscopically, the sperm showed high mass movement (++ – +++), and motility averaged 86.66 ± 4.71%, suggesting strong sperm vigor. This motility level is notably higher than that reported in several Indonesian local chicken breeds such as Kampung (70–80%), Merawang (65–75%), and Kokok Balenggek (Junaedi et al. 2024; Hidayat et al. 2021; Ananda et al. 2024; Husmaini et al. 2024), and aligns with the upper range reported for high-performing native roosters (Rashid and Khalid 2023; Mussa et al. 2023).

Sperm viability was also high, reaching 92.91 ± 3.66%, while the percentage of sperm with intact plasma membranes (IPM) was 91.41 ± 3.57%, reinforcing the excellent physiological integrity of the sperm cells. These findings are in agreement with studies on Kokok Balenggek roosters, which reported viability values around 93% (Jaswandi et al. 2023; Ananda et al. 2025). The relatively low abnormality rate of 8.91 ± 1.60% indicates minimal morphological defects, comparable to those found in other high-fertility local breeds (Ananda et al. 2023; Jaswandi et al. 2025).

Sperm concentration in this study was 2247.66 ± 261.56 million/ejaculate, a value considered optimal for artificial insemination programs. According to Iorio et al. (2020) and Jaswandi et al. (2023), a dose of 200–400 million spermatozoa is typically sufficient per insemination in poultry, which means this volume can yield multiple doses per collection. This finding highlights the fertility potential of Bangkok roosters as breeding sires.

Overall, the fresh semen quality parameters observed here confirm that Bangkok roosters possess strong reproductive potential, with values that are comparable or superior to those of other indigenous breeds studied across Southeast Asia (Sidiqi et al. 2023; Madeddu et al. 2024). The data also support their suitability for short-term semen storage and artificial insemination strategies aimed at genetic conservation or productivity enhancement.

Post-Dilution Semen Quality in Vitamin C–Enriched Citrate Extender

The dilution of Bangkok rooster semen using egg yolk citrate extender supplemented with different concentrations of vitamin C significantly influenced post-dilution sperm quality parameters, including motility, viability, abnormality, and plasma membrane integrity ($P<0.01$). A notable interaction was observed between vitamin C dosage and storage time, with optimal results detected in the 0.2 g/100 mL group (Figure 1).

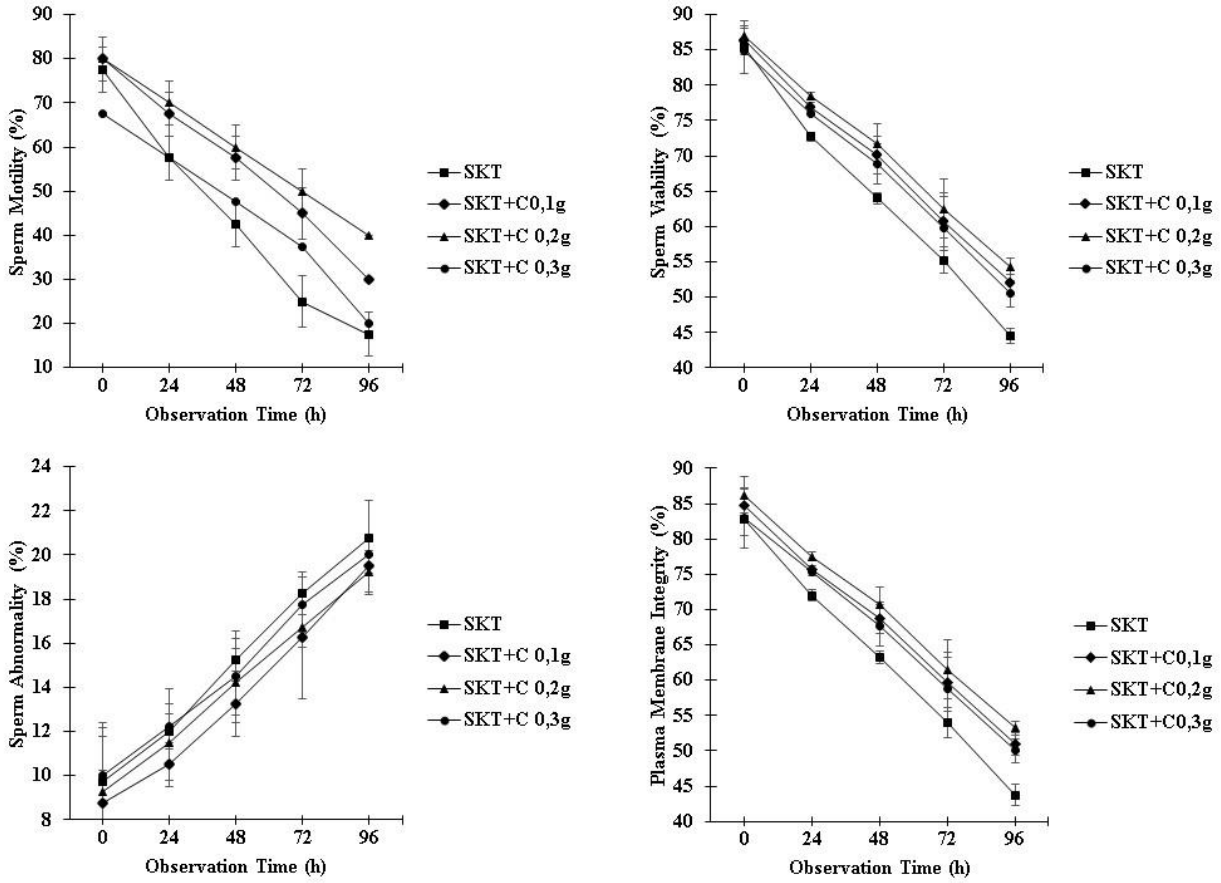

Figure1. The post-dilution semen quality of Bangkok rooster spermatozoa in citrate yolk extender supplemented with different concentrations of vitamin C (0, 0.1 g, 0.2 g, and 0.3 g per 100 mL) stored at 5°C was evaluated based on sperm motility, viability, abnormality, and plasma membrane integrity over 96 h.

Sperm motility declined progressively across all groups during the 96 h storage period. However, the group supplemented with 0.2 g vitamin C maintained the highest motility at each observation point, with 40.00 ± 0.00% at 96 h, significantly higher than the control (17.50 ± 5.00%) ($P<0.01$). These findings align with previous reports indicating the antioxidant properties of vitamin C can delay motility decline by scavenging reactive oxygen species (ROS), which otherwise disrupt mitochondrial activity and flagellar movement (Akhter et al. 2023; Fesahat et al. 2022; Moeinian et al. 2024). A similar effect has been noted in poultry studies, where vitamin C extended sperm motility duration during chilled storage (Uzochukwu et al. 2020; Hidayat et al. 2020).

Sperm viability also decreased over time, but extenders containing vitamin C significantly slowed this decline. The 0.2 g group maintained 54.38 ± 1.11% viability at 96 h, outperforming the control (44.50 ± 1.08%). Vitamin C's protective role in maintaining cell viability is likely attributed to its capacity to stabilize plasma membranes and minimize lipid peroxidation (Sengupta et al. 2022; Najafi et al. 2023). Studies have shown that vitamin C enhances antioxidant enzyme activity, which reduces ROS-induced apoptosis in sperm cells (Hamidian et al. 2020; Hussain and Gaur 2024).

Sperm abnormality increased with storage time in all treatments, but the 0.2 g group consistently showed lower percentages of abnormal sperm compared to other treatments (19.25 ± 0.96% at 96 h), indicating vitamin C's role in preserving morphological integrity. Oxidative stress has been linked to increased sperm deformities, especially in the midpiece and head regions, due to damage to lipids and DNA (Zhang et al. 2022; Ammar et al. 2020). Vitamin C is known to protect nuclear and acrosomal

structures from oxidative fragmentation, thus reducing the occurrence of teratozoospermia (Walczak-Jędrzejowska et al. 2013; Gual-Frau et al. 2015).

Plasma membrane integrity (PMI) was significantly influenced by vitamin C supplementation. At 96 h, the 0.2 g group retained 53.25 ± 0.96% intact membranes, compared to only 43.75 ± 1.50% in the control. The integrity of the plasma membrane is critical for maintaining sperm functionality, as it regulates ion exchange and protects against osmotic stress (Partyka and Niżański 2021). Vitamin C's ability to reduce lipid peroxidation and support membrane fluidity enhances PMI during storage (Prete et al. 2022; Alagbonsi and Olayaki 2020).

Collectively, these findings demonstrate that the addition of 0.2 g vitamin C per 100 mL extender provides the most effective protection for spermatozoa during cold storage. Higher doses (0.3 g) exhibited reduced performance, potentially due to pro-oxidant effects when antioxidant levels surpass cellular requirements (Amini et al. 2015; Ross et al. 2010). These results underscore the importance of optimizing antioxidant concentration in extenders to maintain sperm quality over time.

Post-Dilution Longevity of Bangkok Rooster Spermatozoa in Vitamin C–Enriched Citrate Extender

The longevity of spermatozoa stored at 5°C showed a significant response to vitamin C supplementation in the citrate extender (Figure 2). The highest mean longevity was observed in the SKT + 0.2 g treatment group, with an average duration of 11.25 ± 0.96 days, significantly longer ($P<0.05$) than the control group (SKT) with only 7.50 ± 0.58 days. This finding indicates that moderate supplementation with vitamin C can effectively extend the viability of rooster sperm under cold storage conditions. In comparison, Ananda et al. (2023) observed that Ringer's Lactate (RL) extender supplemented with egg yolk could sustain Kokok Balenggek rooster sperm motility for up to 6 days at 5°C, while Ananda et al. (2025) showed that RL alone maintained motility for up to 12 days but with gradual quality deterioration. The current study's result, showing 11-day longevity in Bangkok roosters with 0.2 g vitamin C supplementation, aligns with these findings and confirms the efficacy of combining citrate extender with appropriate antioxidant concentrations.

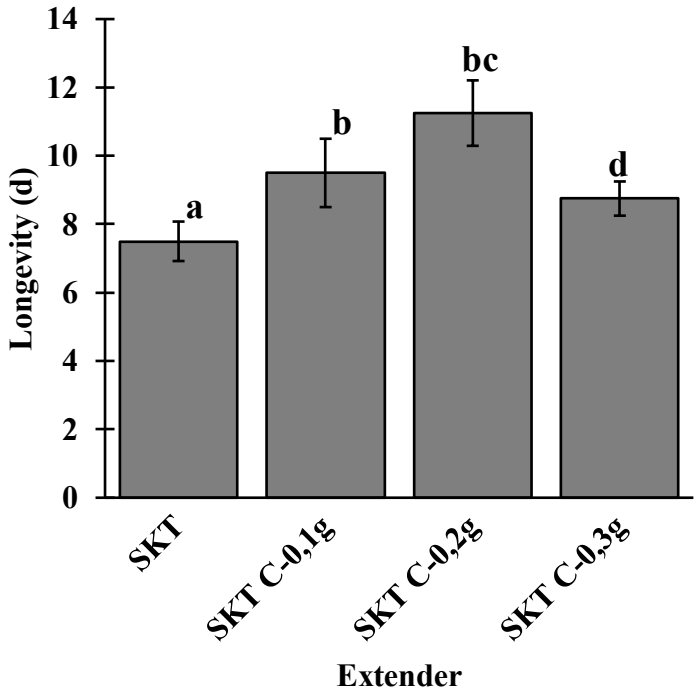

Figure 2. Longevity of Bangkok rooster spermatozoa in citrate yolk extender supplemented with different concentrations of vitamin C (0, 0.1, 0.2, and 0.3 g per 100 mL) stored at 5°C. Different letters indicate significant differences ($P<0.05$) among treatments.

Vitamin C acts as a potent antioxidant that mitigates oxidative damage by scavenging reactive oxygen species (ROS), which are known to accumulate during cold storage and cause lipid peroxidation, DNA fragmentation, and structural deterioration of the sperm membrane (Hamidian et al. 2020; Gual-Frau et al. 2015; Akhter et al. 2023). The preservation of sperm longevity at 0.2 g vitamin C may thus be attributed to the compound's optimal role in maintaining membrane fluidity and reducing oxidative stress during prolonged storage (Zampini et al. 2020; Sahashi et al. 2011).

Interestingly, a decrease in longevity was observed at the highest supplementation level (SKT + 0.3 g), where sperm survived only 8.75 ± 0.50 days, suggesting a potential pro-oxidant effect at high doses. This phenomenon has been previously documented in antioxidant research, where excessive levels of vitamin C may paradoxically lead to increased oxidative activity via Fenton reactions, especially in the presence of transition metals (Fisher and Naughton 2004; Harvey et al. 2007). The hormetic nature of antioxidants such as vitamin C indicates that balance is critical, as both deficiency and excess can negatively impact cellular function (Hirata et al. 2020).

These results support earlier reports emphasizing the importance of antioxidant-enriched extenders for improving sperm longevity in avian species (Hayanti et al. 2022; Vicente et al. 2018). Antioxidants in extenders not only stabilize mitochondrial function and reduce malondialdehyde (MDA) production, but also help maintain ATP levels essential for flagellar movement, which is crucial for prolonged motility (Perumal et al. 2014; Akhter et al. 2023). Moreover, increased sperm longevity enhances the probability of successful fertilization, particularly in artificial insemination programs that rely on extended sperm usability (Gasparini et al. 2010). In poultry, this translates into improved fertility rates, especially in environments with limited male access or synchronized ovulation cycles.

Overall, this study demonstrates that 0.2 g vitamin C per 100 mL extender provides an optimal concentration for extending sperm longevity in Bangkok roosters under chilled storage. This finding is particularly relevant for the design of extender formulations aimed at improving reproductive efficiency in native chicken breeding programs.

CONCLUSION

Supplementation of 0.2 g/100 mL vitamin C in citrate egg yolk extender significantly improved sperm motility, viability, membrane integrity, and longevity of Bangkok rooster semen stored at 5°C compared to the control. The longevity extended up to 11.25 days, indicating better preservation. However, higher doses showed no added benefit and may reduce longevity. This finding suggests that 0.2 g/100 mL is an optimal dose for semen storage and can support the success of artificial insemination programs in poultry. Future studies should examine in vivo fertility outcomes and potential synergistic effects with other antioxidants.

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