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Motility and Viability of Kokok Balenggek Chicken Spermatozoa in Various Commercial Physiological Solutions Stored at 4ºC

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ABSTRACT

This study investigates the motility and viability of Kokok Balenggek chicken (KBC) spermatozoa in various commercial physiological solutions, specifically Ringer's Lactate (RL), Sodium Chloride 0.9% (NaCl 0.9%), and Phosphate Buffered Saline (PBS), all stored at 4ºC. A total of 15 KBC specimens were utilized in this research. Semen collection occurred bi-weekly at 3-day intervals, followed by a comprehensive evaluation of spermatozoa quality, encompassing volume, pH, color, consistency, mass movement, motility, concentration, and viability. The results indicated that the average volume of KBC semen was 0.35±0.16 mL, characterized by a white color, thick consistency, and a pH of 7.09±0.21. Microscopic evaluation unveiled a spermatozoa mass movement score of 3.0±0.0 (+++), spermatozoa motility of 90±0.0%, live spermatozoa percentage of 95±1.71%, and a spermatozoa concentration ranging from 2419±0.74×10^6 cells/mL. Moreover, a significant difference ($P<0.05$) in the longevity of KBC spermatozoa was observed in RL solution compared to 0.9% NaCl solution and PBS. RL diluent exhibited superior performance ($P<0.05$) over 0.9% NaCl solution and PBS, with the longest motility and viability persisting up to 288 hours (0.71±1.82% and 0.84±2.22%). Conversely, the 0.9% NaCl solution displayed the shortest motility and viability, lasting 72 hours (1±2% and 2±4%), while the PBS solution maintained viability up to 120 hours (0.3±1.3% and 0.5±1.9%). In conclusion, storing KBC semen at 4ºC using RL diluent resulted in superior longevity, motility, and viability of spermatozoa compared to 0.9% NaCl solution and PBS.

Key words: KBC, Semen quality, Longevity, Motility, Viability

INTRODUCTION

The Kokok Balenggek chicken (KBC), an indigenous breed from West Sumatra, Indonesia, is renowned for its unique crowing pattern and superior meat quality, attracting significant attention for its potential to enhance local poultry production (Husmaini et al. 2024). Thriving in the Payung Sakaki District, Solok Regency, this breed has potential applications beyond meat and egg production, serving roles as ornamental, fighting, and "singing" chickens due to their distinctive crow, locally termed "Balenggek." As valuable germplasm of the Minang Realm, the preservation and development of KBC are imperative. Selective breeding holds the promise of producing superior local broiler chickens (Husmaini et al. 2023). As of 2021, the KBC population reached 1,960 individuals, with a male-female ratio of 1:1.3 (Husmaini et al. 2022). Previous reports indicated male-female ratios of 1:7 for in situ conservation, with no specification for ex situ conservation (Rusfidra et al. 2014; Rusfidra 2015). These findings underscore the significance of ongoing conservation efforts for this unique and culturally significant breed, known for its robust adaptability and disease resistance compared to purebred counterparts.

Understanding the qualitative and quantitative characteristics of Kokok Balenggek chickens is essential for developing superior local breeds. Husmaini et al. (2023) investigated the formation of superior local meat-type chickens by evaluating the G0 generation of Kokok Balenggek chickens. Their findings provided insights into the breed's potential for producing high-quality meat, crucial for meeting local consumer demands. Furthermore, Husmaini et al. (2024) explored the hatching performance of the G1 generation of Kokok Balenggek chickens, highlighting the importance of optimizing hatching conditions to improve overall performance.

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Artificial insemination (AI) has become crucial for improving the reproductive efficiency of Kokok Balenggek chickens. AI, widely adopted in livestock, plays a key role in elevating poultry quality and supporting population growth. This technique often involves using liquid or frozen semen from selected superior males, enabling long-term storage and subsequent insemination into female birds. Jaswandi et al. (2023) reported that factors such as the quality of rooster spermatozoa significantly influence the fertility rate, fertility period, and day-old chick (DOC) sex ratio after AI. Successful semen storage, both short-term (liquid semen) and long-term (frozen semen), requires optimal conditions. For short-term storage at low temperatures, dilution is a common approach to increase semen volume and prevent rapid quality deterioration. The diluent must provide necessary nutrients to sustain semen viability during storage (Iswati et al. 2021). An ideal diluent includes ample energy sources, buffering agents, cold shock ingredients, protective measures against bacterial contamination, and must be non-toxic to spermatozoa, supplying essential energy substances to support spermatozoa vitality (Najafi et al. 2022).

The motility and viability of rooster spermatozoa are essential factors in AI and breeding programs. Various solutions and extenders have been researched to optimize storage conditions for rooster spermatozoa at chilled temperatures. Studies have explored the effects of different additives such as egg yolk, trehalose, coconut water, and antioxidants on sperm quality during storage. For instance, Azzam et al. (2022) investigated using a 5% dextrose Ringer's solution and egg yolk extender to maintain the motility and viability of kampung rooster spermatozoa at 5ºC. Similarly, Diba et al. (2023) focused on the impact of adding egg yolk to a physiological saline extender on sperm quality at cool temperatures. These studies emphasize the significance of extender composition in preserving rooster spermatozoa. Additionally, variations in sperm quality and fecal testosterone levels among different phenotypes of Kokok Balenggek roosters have been documented, further emphasizing the breed's genetic diversity (Ananda et al. 2024).

Commonly accessible diluents for poultry semen include physiological NaCl, dextrose, Ringer's, Lockes, Tyrodes, and Lifos, as well as buffer media like PBS, TALP, and Tris. For more intricate requirements, commercial poultry semen diluents such as Lake's diluent, Beltsville Poultry Semen Extender (BPSE), Instruments for Veterinary Medicine (IMV), and CARI are used, primarily for frozen semen storage in poultry (Hudson et al. 2016). While these commercial diluents offer advanced features, their importation and high cost necessitate affordable alternatives for farmers. A standard and economical option is NaCl 0.9%, a physiological solution known to maintain the metabolic activity of animal cells (Iswati et al. 2021). Previous research notes that semen quality using a 0.9% NaCl diluent typically lasts around 30-45 minutes at room temperature (Lubis 2011). Alternatively, Ringer Lactate (RL), with its enhanced buffering and isotonic composition, has been identified as a material supporting semen survival for an extended duration, up to 18 hours after ejaculation (Danang et al. 2012). Phosphate Buffered Saline (PBS), a commonly used physiological solution in biological research, serves as a solvent due to its isotonic and non-toxic nature, maintaining osmolarity (Haq et al. 2020). Including antibiotics in diluents is crucial to prevent pathogenic bacterial growth that could jeopardize chicken spermatozoa. These insights contribute to developing practical and cost-effective semen dilution methods for sustainable poultry breeding practices.

The three materials (NaCl 0.9%, RL, and PBS) constitute commercial semen diluents that are simple, readily available, and economically viable, featuring distinct ion and mineral compositions. They hold promise for utilization as poultry semen diluents. To identify the optimal formulation for frozen chicken semen diluent, fundamental research is imperative, involving a comprehensive study of sperm conditions within the diluent at 4ºC for liquid semen. Hence, this study aims to compare the quality of Kokok Balenggek chicken (KBC) spermatozoa in 0.9% NaCl, RL, and PBS diluents stored at 4ºC for 12 hours. The longevity and motility of Kokok Balenggek rooster spermatozoa have been extensively studied. Ananda et al. (2023) demonstrated that sperm motility could be sustained when stored in Ringer's lactate solution supplemented with egg yolk, highlighting the importance of the storage medium in preserving sperm quality.

Despite extensive research on Kokok Balenggek chickens, limited information is available on the effects of different commercial physiological solutions on the motility and viability of rooster spermatozoa stored at low temperatures. This study aims to fill this gap by evaluating the motility and viability of Kokok Balenggek rooster spermatozoa in various commercial physiological solutions stored at 4ºC. Understanding the optimal storage conditions for rooster spermatozoa will contribute to the success of AI programs and the conservation of this valuable indigenous breed. The objective is to determine the minute, hour, or day until the spermatozoa maintain suitable quality for AI. The study also involves assessing the motility and viability of KBC spermatozoa from the three commercial diluents at 4ºC.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committee of Universitas Andalas, West Sumatera, Indonesia.

Semen collection, evaluation, and dilution

This study used 15 KBC aged 24 months with a body weight of 1.8±0.2 kg. During the study, each KBC was intensively maintained in a battery cage (thick wire material) measuring 35×56×35 cm. The feed provided was commercial feed (ABS Crumble, PT. Japfa Comfeed Indonesia), given *ad libitum*. Before semen collection, chickens were fasted to prevent feces excretion during the procedure.

Semen was collected twice a week (with a 3-day interval between collections) in the afternoon from 17:30 to 18:00 using the massage method. Collected semen was placed in a 1.5 mL microtube and immediately taken to the laboratory for evaluation. Evaluation of KBC spermatozoa quality included both macroscopic and microscopic tests.

Macroscopic quality evaluation included volume (µL), color, consistency, and pH, while microscopic quality evaluation included spermatozoa mass movement, spermatozoa motility (%), spermatozoa viability (%), and

spermatozoa concentration (10^6/ejaculate). Fresh semen, after quality testing, was immediately given a diluent according to the treatment (commercial diluent solution). The diluents used were 0.9% NaCl, Ringer's Lactate (RL), and Phosphate Buffered Saline (PBS). Semen was diluted to a concentration of 2×10^8 cells/mL. Each diluted semen sample was then placed in a 1.5mL microtube (Fig.1).

Storage, observation of sperm motility and viability

Semen diluted with commercial diluent solutions (0.9% NaCl, RL, and PBS) was stored at 4°C. The motility and viability of the diluted spermatozoa were observed every 12 hours until sperm motility reaches 0%. Sperm motility was observed using a microscope with a magnification of 400×, assessed from five fields of view, and expressed as a percentage. Sperm viability was observed using a microscope with a magnification of 400× by preparing a smear with a staining solution. Viability was assessed by counting at least 200 individuals per slide; dead spermatozoa were indicated by absorbing the stain, while live spermatozoa remain transparent. The percentage of chicken spermatozoa motility was determined by comparing the estimated number of motile spermatozoa to the total number of spermatozoa visible in the observation. Chicken spermatozoa viability was calculated by comparing the number of live spermatozoa to the total number of spermatozoa observed, then multiplying by 100%.

Data analysis

Motility and viability data obtained from observations every 12 hours were analyzed descriptively and quantitatively. Meanwhile, semen motility and viability data observed at 0h, 12h, 24h, and 36h were analyzed using analysis of variance (ANOVA) with the SPSS version 25 for Windows application. Differences between treatment groups were further analyzed using the Duncan test.

RESULTS

Fresh semen quality

The results of the study showed that the evaluation of fresh KBC semen after collection was feasible for dilution (Table 1). The evaluation of semen quality involved both macroscopic and microscopic tests. The results of the macroscopic semen evaluation showed an average KBC semen volume of 0.35±0.16 mL, with a white color, thick consistency, and a pH of 7.09±0.21. Meanwhile, the results of the microscopic semen evaluation showed a spermatozoa mass movement of 3.0±0.0 (+++), spermatozoa motility of 90±0.0%, a live spermatozoa percentage of 95±1.71%, and a spermatozoa concentration of 2419±0.74×10^6 cells/mL.

Table 1: Fresh semen quality of Kokok Balenggek rooster

No	Parameters	Average
1	Volume (mL)	0.35±0.16
2	Color	White
3	Consistency	Thick
4	pH	7.09±0.21
5	Mass movement	3±0.0
6	Concentration (10^6 cells/mL)	2419±0.74
7	Motility (%)	90±0.0
8	Viability (%)	95±1.71

n=15

Longevity of spermatozoa in commercial physiological solutions stored at 4ºC

The results of the study (Fig. 2) showed that the average longevity of motility and viability of KBC spermatozoa differed in each diluent solution (0.9% NaCl, RL, and PBS). The RL diluent had the longest shelf life (159.2±66.88 hours), while the 0.9% NaCl diluent had the shortest shelf life (60±7.86 hours). The PBS diluent (77.6±22.16 hours) had a higher shelf life than the 0.9% NaCl solution but lower than the RL solution. The results also showed (Fig. 2) that the longevity of KBC spermatozoa in the RL solution had a significant difference ($P<0.05$) compared to the 0.9% NaCl solution and PBS. In contrast, the 0.9% NaCl solution did not show a significant difference ($P>0.05$) compared to the PBS solution.

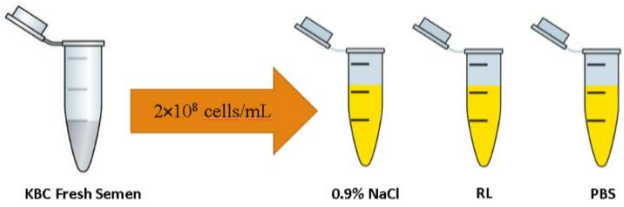

Fig. 1: Research Design.

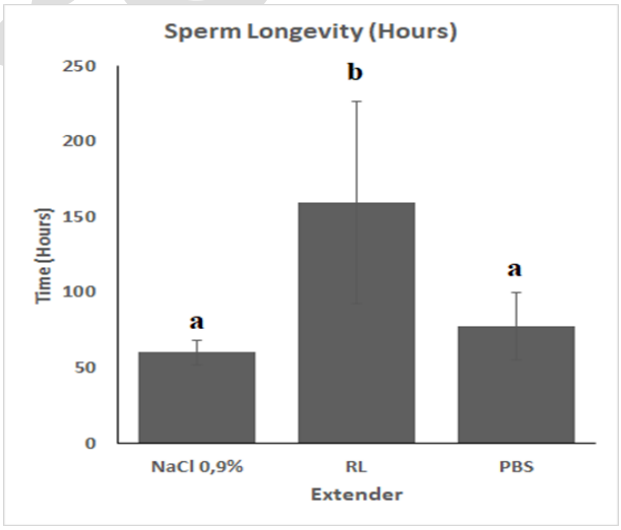

Fig. 2: Diagram of spermatozoa longevity stored at 4ºC with NaCl 0.9%, RL, and PBS diluents, observed every 12 hours.

Motility of spermatozoa in commercial physiological solutions stored at 4ºC

The results showed that the motility of KBC spermatozoa (Fig. 3) in 0.9% NaCl solution, Ringer's Lactate (RL), and Phosphate Buffered Saline (PBS) stored at 4°C decreased with the storage period. The percentage decrease every 12 hours of storage ranged from 0% to 30%. The results indicated that the RL diluent was more effective than 0.9% NaCl solution and PBS, with the longest motility reaching 288 hours (0.71±1.82%). In contrast, the 0.9% NaCl solution had the shortest motility duration of 72 hours (1±2%). The PBS solution lasted up to 120 hours (0.3±1.3%), which was higher than the 0.9% NaCl solution but lower than the RL solution.

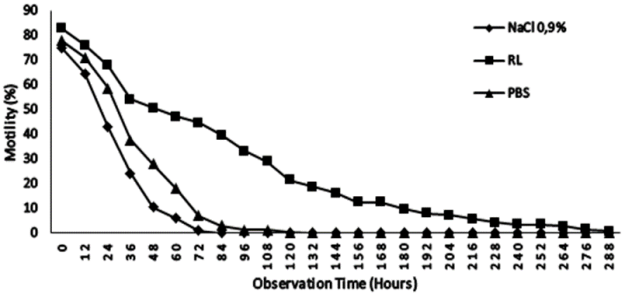

Fig. 3: Line graph of spermatozoa motility stored at 4ºC with NaCl 0.9%, RL, and PBS diluents, observed every 12 hours.

The results of observations on the motility of KBC spermatozoa at 0h, 12h, 24h, and 36h were based on semen quality (motility of KBC spermatozoa) in the range of ≥40%. The results showed (Fig. 4) that at 0h and 12h, there was no significant difference (P>0.05) between the use of 0.9% NaCl diluent, RL, and PBS. However, at 24h and 36h, there was a decrease in motility, resulting in a significant difference (P<0.05) between the RL solution and the NaCl and PBS solutions. There was no significant difference between the PBS solution and the NaCl solution.

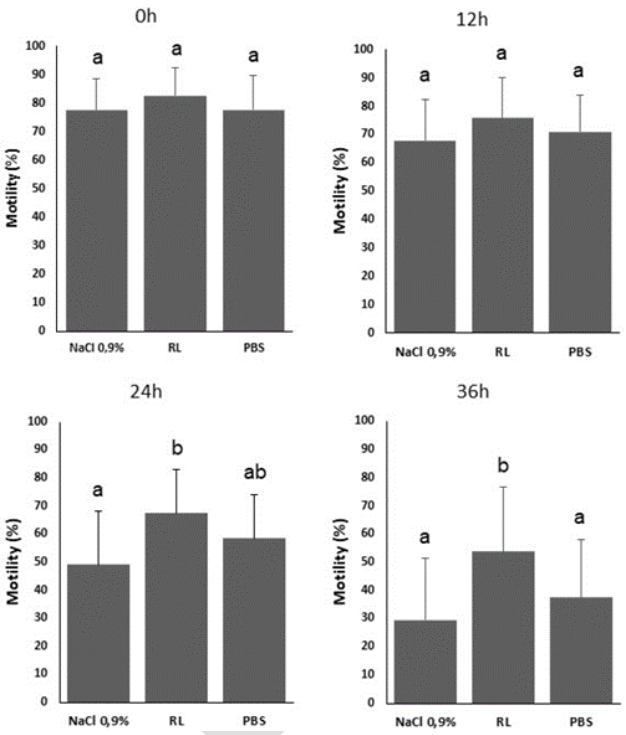

Fig. 4: The diagram of spermatozoa motility stored at 4ºC with NaCl 0.9%, RL, and PBS diluents, observed at 0h, 12h, 24h, and 36h.

Viability of spermatozoa in commercial physiological solutions stored at 4ºC

The results showed that the viability of KBC spermatozoa (Fig. 5) in 0.9% NaCl solution, Ringer's Lactate (RL), and Phosphate Buffered Saline (PBS) stored at 4°C decreased over the storage period. The percentage decrease in KBC spermatozoa viability every 12 hours of storage ranged from 0% to 20%. Observations made until the viability percentage reached the lowest value showed that the use of RL diluent was better than 0.9% NaCl solution and PBS, with a storage period of 288 hours (0.84±2.22%). Meanwhile, 0.9% NaCl solution in viability observations showed a lower storage period of 72 hours (2±4%). In PBS solution, viability lasted up to 120 hours (0.5±1.9%), which was higher than 0.9% NaCl solution but lower than RL solution.

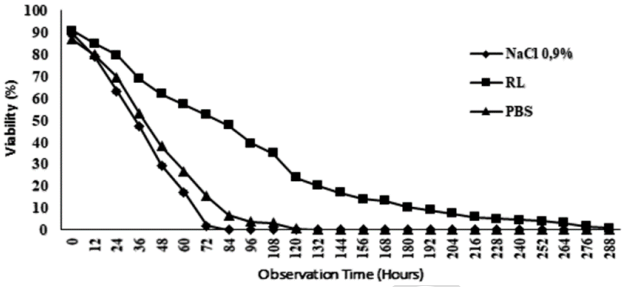

Fig. 5: Line graph of spermatozoa viability stored at 4ºC with NaCl 0.9%, RL, and PBS diluents, observed every 12 hours.

The results of observations on the viability of KBC spermatozoa (Fig. 6) were not much different from observations of spermatozoa motility at observation times 0h, 12h, 24h, and 36h. Observations at 0h and 12h showed no significant difference (P>0.05) between the use of 0.9% NaCl diluent, RL, and PBS. However, at 24h and 36h, a decrease in viability was seen, resulting in a significant difference (P<0.05) between the RL solution and the NaCl and PBS solutions. There was no significant difference between the PBS solution and the NaCl solution. Observations on the viability of KBC spermatozoa during the storage period (0h, 12h, 24h, and 36h) were also carried out based on semen quality (KBC spermatozoa viability) which was in the range of (≥40%).

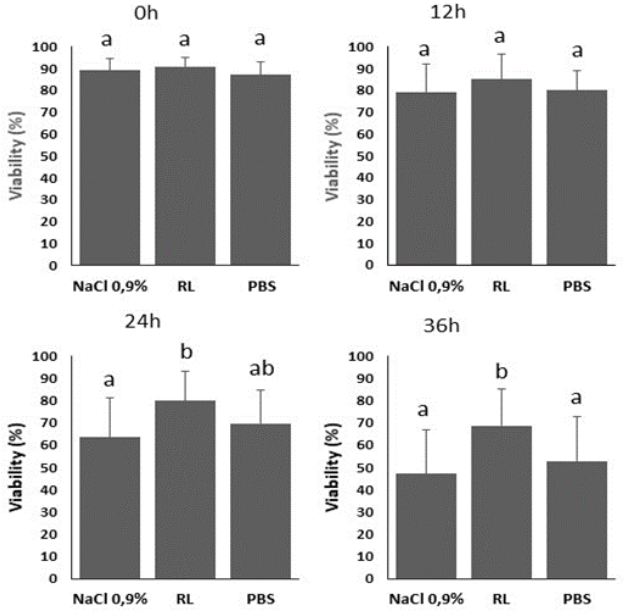

Fig. 6: The diagram of spermatozoa viability stored at 4ºC with NaCl 0.9%, RL, and PBS diluents, observed at 0h, 12h, 24h, and 36h.

DISCUSSION

Fresh semen quality of KBC

Fresh semen quality is a critical factor in poultry production, particularly when considering specific chicken breeds like the Kokok Balenggek. Research has

demonstrated that assessing parameters such as motility, viability, concentration, and pH of fresh semen is crucial for understanding the reproductive potential of roosters (Mussa et al. 2023). Observation of spermatozoa evaluation is crucial for determining the suitability of spermatozoa for research purposes. In this study, the average volume of KBC semen was 0.35±0.16 mL/ejaculate (Table 1). This result is higher than that reported by Ananda et al. (2023) and Ananda et al. (2024), who indicated a volume of 0.2-0.3 mL/ejaculate, but lower than the findings of Jaswandi et al. (2023), who recorded a volume of 0.415mL, and Magfira et al. (2017), who reported a volume of 0.59mL for Merawang chicken. Variations in semen volume among different chicken breeds can be attributed to normal physiology, spermatogenesis processes, and responses to massage techniques during semen collection (Ayeneshet et al. 2024).

The color and consistency of KBC semen in this study were considered normal, being white and thick. These results align with previous findings where KBC semen was also described as milky white and thick (Ananda et al. 2023; Jaswandi et al. 2023; Ananda et al. 2024). Semen quality can be partially assessed through its color and consistency (Almahdi et al. 2014). The fresh semen in this study had an average pH of 7.09±0.21, indicating normalcy. Octa et al. (2014) stated that sperm suitable for research should have a cloudy white color, a distinctive aroma, thick consistency, and a pH above 6.7.

The average concentration of KBC semen in this study was 2419±0.738×10^6 cells/ejaculate. This is higher than the concentration reported by Ananda et al. (2023) and Ananda et al. (2024), which ranged from 817-1347×10^6 cells/ejaculate, and higher than the findings for Pelung chicken (1160±11.73×10^6 cells/ejaculate) by Junaedi and Husnaeni (2019), and for Green Jungle Chicken (898±4×10^6 cells/ejaculate) by Bebas and Laksmi (2013). However, it was lower than the concentration reported by Jaswandi et al. (2023), which was 2432.34±668.08×10^6 cells/ejaculate.

The average motility of KBC spermatozoa in this study was found to be 90±0.0%. This percentage is higher compared to the 70.63±1.36% reported by Ananda et al. (2023) and 79±16.9% by Ananda et al. (2024) for KBC. The 84.69±1.12% reported for Pelung chicken by Junaedi et al. (2016), and the 86% reported by Kusuma et al. (2018). The criteria for fresh semen that can be processed for dilution are met by the motility of KBC spermatozoa in this study. Factors influencing the motility of chicken spermatozoa, including genetics and the subjective assessment of the observers (Udrayana et al. 2023). According to Danang et al. (2012), the forward movement of spermatozoa is essential to reach the site of fertilization in the female genital tract. Fresh semen in this study had a spermatozoa viability percentage of 95±1.71%, which is considered good according to Arifiantini (2012), who stated that good spermatozoa viability ranges between 60-75%. This study's results are higher than those reported by Ananda et al. (2023), Jaswandi et al. (2023), and Ananda et al. (2024) for KBC, which were 79.4±1.04%, 93.06±5.61%, and 93.3±4.2% respectively. Viability is a crucial factor in assessing spermatozoa quality, as it plays a significant role in the success of fertilization, particularly in insemination.

Various factors, including age, diet, and environmental conditions, can significantly affect semen quality in roosters (Haryuni et al. 2022; Nemati et al. 2023; Liang et al. 2024). For example, aging has been found to influence sperm quality, with roosters of specific age ranges producing higher quality spermatozoa (Haryuni et al. 2022). Furthermore, dietary supplements like ginger have been researched for their potential effects on testicular histology, semen characteristics, and reproductive performance in breeder roosters (Nemati et al. 2023). Dietary restrictions have also been associated with promoting sperm remodeling in aged roosters, highlighting the complex relationship between nutrition and semen quality (Liang et al. 2024).

The utilization of organic trace mineral premixes has been linked to enhanced semen quality in male broiler breeders, indicating a connection between nutrition and reproductive performance (Shan et al. 2017). These findings underscore the significance of considering dietary factors and supplements to manage and improve semen quality in poultry breeding programs.

Understanding the various factors that influence fresh semen quality in roosters, such as genetic traits, dietary supplements, and environmental conditions, is crucial for optimizing reproductive success in poultry production, including breeds like the Kokok Balenggek. This study has provided valuable insights into the semen quality parameters of KBC, contributing to the broader understanding of reproductive physiology in poultry.

Longevity of KBC spermatozoa in commercial physiological solutions stored at 4ºC

The storage time factor has been shown to affect spermatozoa motility, meaning that the longer the storage time, the lower the quality of spermatozoa (AL-Saeedi Tahseen et al. 2019). Sperm longevity is measured by observing how long it remains motile. During storage, the quality of KBC spermatozoa in this study decreased in each diluent (0.9% NaCl, RL, and PBS). These results are consistent with the report of Trummer et al. (1998), which stated that storage time can cause a decrease in spermatozoa motility. In this study, observations of motility and viability were carried out every 12 hours at a temperature of 4ºC (Fig. 3 and 4). The longest longevity was found in the RL solution ($P<0.05$), which had the longest shelf life, averaging 159.2±66.88 hours. Meanwhile, the shortest shelf life was found in the 0.9% NaCl solution ($P>0.05$), averaging 60±8.15 hours. The PBS solution ($P>0.05$) had an average shelf life of 77.6±22.16 hours.

Research on the longevity of KBC semen has been previously conducted with the addition of several diluents by Ananda et al. (2023), which resulted in an average longevity of KBC spermatozoa of 5.03–5.93 days. Other studies on longevity in local chickens have also been widely reported: kampung chickens with a shelf life exceeding 4.5 days (Indrawati et al. 2013) and 6.7 days (Hardiyanti and Kurniawan 2020), and Merawang chickens with a lifespan between 4.43 and 5.93 days (Magfira et al. 2017). According to (Bozkurt et al. 2007), the longevity of spermatozoa is influenced by the concentration of spermatozoa and the type of diluent used. Longer semen storage reduces spermatozoa motility and vitality. During

prolonged storage of chicken semen, spermatozoa damage often occurs due to the respiration process in spermatozoa mitochondria, which produces free radicals (Zong et al. 2023). The cell plasma membrane is most susceptible to damage by free radicals due to its composition of phospholipids and glycolipids containing unsaturated fatty acids. Free radicals take electrons from unsaturated fatty acids (Martemucci et al. 2022), resulting in damage to all phospholipids in the spermatozoa plasma membrane. Damage to the spermatozoa plasma membrane can interfere with the active transfer of substances that are a source of energy for spermatozoa, leading to decreased spermatozoa vitality (Partyka and Niżański 2021). Further findings by Helfenstein et al. (2010) showed that the length of the spermatozoa tail also affects longevity, where spermatozoa with long tails tend to have high motility but a shorter lifespan, while spermatozoa with short tails can have a long lifespan even though their motility is low.

To investigate the longevity of Balenggek chicken's Kokok spermatozoa using commercial physiological solutions (0.9% NaCl, PBS, and Ringer's lactate) stored at 4ºC, it is essential to consider the impact of different extenders on sperm quality and longevity. Extenders play a crucial role in preserving sperm viability and motility during storage. Studies have shown that the composition of extenders, such as Ringer's lactate-egg yolk diluent (Ananda et al. 2023), lactated Ringer's-egg yolk with additives like melon flesh juice (Pitaloka et al. 2023), and Biladyl with Equex STM paste or Andromed (Nöthling et al. 2007), can influence spermatozoa plasma membrane integrity, morphological abnormalities, and motility.

Furthermore, the addition of substances like caffeine, pentoxifylline, and 2′-deoxyadenosine has been shown to stimulate ejaculated sperm motility (Stachecki et al. 1995). Additionally, antioxidants like GSH-Px and CAT have been linked to improved motility and membrane integrity of spermatozoa (Pagl et al. 2006). These findings suggest that the choice of extender and additives can significantly impact sperm quality during storage. Moreover, studies on other animal species, such as Landrace boar (Adu et al. 2023), snow leopards (Roth et al. 1994), and sea urchins (Binet and Doyle 2013), have highlighted the importance of extender composition and storage conditions in maintaining sperm longevity. For instance, the use of specific diluents like BTS + 3% SDMLE has been shown to enhance sperm quality (Adu et al. 2023).

The longevity of Kokok Balenggek chicken's spermatozoa stored at 4ºC using commercial physiological solutions can be influenced by the choice of extender, additives, and storage conditions. Extenders like Ringer's lactate-egg yolk diluent, lactated Ringer's-egg yolk with melon flesh juice, and Biladyl with Equex STM paste have shown promising results in maintaining sperm quality. Understanding the effects of different extenders and additives is crucial for optimizing the storage and preservation of spermatozoa for future breeding programs.

Motility of KBC spermatozoa in commercial physiological solutions stored at 4ºC

The results of the study showed (Fig. 3) that over time, the motility of KBC spermatozoa in 0.9% NaCl diluent, RL, and PBS at a temperature of 4ºC decreased. The percentage of motility decreases every 12 hours of storage ranged from 0 to 30%. In this study, storing KBC semen in 0.9% NaCl diluent, RL, and PBS resulted in spermatozoa motility suitable for insemination at 0h, 12h, 24h, and 36h. Semen stored for 0-36 hours had an average motility of ≥40%. This is in accordance with the statement of Solihati et al. (2006), which states that the lowest motility that semen must have to perform artificial insemination is 40%.

Observations at 0h and 12h did not show a significant difference ($P>0.05$) between the use of 0.9% NaCl diluent, RL, and PBS. These results are consistent with the research conducted by Khaeruddin and Amir (2019), which stated that the use of various types of extenders had no effect ($P>0.05$) on the progressive motility of male sperm stored at 0 hours or at 24 hours of storage. However, at 24h and 36h, there was a decrease in motility which caused a significant difference ($P<0.05$) between RL solution with 0.9% NaCl and PBS (Fig. 4). However, there was no significant difference between PBS solution and 0.9% NaCl solution.

Storing semen for a longer time causes a decrease in sperm motility because the energy supply is increasingly limited. During storage, spermatozoa remain active in moving and metabolizing. Longer storage times also cause a decrease in pH due to spermatozoa metabolism, which takes place both aerobically and anaerobically. Fig. 3 shows that the use of RL diluent gives better results than 0.9% NaCl solution and PBS, with the longest motility reaching 288 hours (0.71±1.82%). These results are the same as the study conducted by Iswati et al. (2021), which stated that the motility of native chicken spermatozoa in RL diluent is better than in physiological NaCl diluent. This is thought to be due to the content in Ringer lactate diluent. One of the components in Ringer lactate diluent is Na-lactate, which is able to meet the needs of bicarbonate ions as a buffer solution and maintain osmotic pressure in spermatozoa. The high effectiveness of Ringer lactate diluent is due to the presence of minerals such as potassium and sodium, which can protect the membrane in chicken spermatozoa (Triadi et al. 2022). These results are in line with the study of Khaeruddin et al. (2020), which stated that Ringer lactate contains sodium and potassium minerals that play a role in the process of active transport of substances that pass through the cell membrane. Danang et al. (2012) added that the use of Ringer lactate solution can maintain the sustainability of sperm quality after semen collection.

Sperm motility is closely related to spermatozoa viability, meaning that a low percentage of motility will result in a low percentage of viability. Conversely, high motility will result in high viability. This shows that the value of spermatozoa motility has a significant impact on spermatozoa viability. A high percentage of motility reflects progressive movement and mass, indicating that spermatozoa are still alive in large numbers and produce a high percentage of viability after administration of Ringer lactate solution (Fig. 5).

The use of 0.9% NaCl solution showed the shortest spermatozoa motility resistance, which was 72 hours. This is due to the small amount of electrolyte fluid in the 0.9% NaCl solution compared to Ringer lactate or PBS, which function to maintain better motility. PBS solution, as an isotonic physiological solution, shows motility resistance reaching 120 hours. Phosphate buffer in PBS acts as a

solvent that is often used in living cell research because of its nutrient content and buffering ability.

The provision of diluents for a 12-hour storage period at 4ºC has an effect on decreasing the motility of KBC spermatozoa. This is caused by an increase in lactic acid produced from carbohydrate metabolism by spermatozoa, which causes the spermatozoa to become acidic. Carbohydrates that undergo metabolism into lactic acid will release H+ ions in spermatozoa, which causes an increase in H+ ions. If H+ ions meet with air elements, hydrogen peroxide can be formed. Hydrogen peroxide has a high potential for damage because it can damage the structure of the cell membrane by forming lipid peroxide (L-OOH) (Jeong et al. 2016). Damage to the plasma membrane is thought to cause metabolic disorders and suboptimal energy production, resulting in decreased spermatozoa motility (Kaltsas 2023).

Ananda et al (2023) explored the effect of Ringer's Lactate-egg yolk diluent on the longevity and motility of spermatozoa from different phenotypes of Kokok Balenggek Chicken. This study can offer relevant information on the impact of specific diluents on sperm motility. Diep and Blesbois (2018) demonstrated the positive effects of Metformin on chicken sperm quality, including motility. Understanding how additives influence sperm motility can be crucial in evaluating the impact of different physiological solutions on Balenggek chicken's Kokok spermatozoa. Vasicek et al. (2015) and Slanina et al. (2015) investigated the effect of diluents and storage conditions on sperm characteristics in roosters and turkeys, respectively. These studies can provide insights into the role of diluents and temperature on sperm motility, which is pertinent to the current research question. Moreover, Mumtaz et al. (2022) evaluated extenders for the liquid storage of pheasant semen, highlighting the importance of the right diluent in maintaining sperm motility. Understanding the impact of different extenders can aid in selecting the most suitable solution for storing Kokok Balenggek chicken's spermatozoa. By synthesizing findings from studies on sperm motility, diluents, and semen preservation in poultry, valuable insights can be gained to assess the motility of Balenggek chicken's Kokok spermatozoa using Commercial Physiological Solutions stored at 4ºC.

Viability of KBC spermatozoa in commercial physiological solutions stored at 4ºC

Other semen quality factors that determine the success of artificial insemination include spermatozoa viability. Viability is crucial because only spermatozoa that survive in the female reproductive tract can reach the fertilization site and fertilize the ovum. The results of this study indicate that spermatozoa viability decreases with increasing observation time until the end of observation (Fig. 5).

The quality of semen in RL diluents tends to be better than in 0.9% NaCl and PBS. This is due to the more complete content in RL, including Na-lactate compounds, which help spermatozoa survive longer with better viability quality (Azzam et al. 2022). Lactate, a source of energy in RL, is a product of gluconeogenesis from carbohydrate metabolism and sugar breakdown occurring in the cell cytoplasm, producing energy in the form of Adenosine TriPhosphate (ATP). ATP serves as a source of energy for spermatozoa during storage, maintaining their viability (Yumte et al. 2013). The high viability value of Ringer lactate diluent is attributed to its electrolyte content, which aligns with semen plasma and helps maintain the viability of KBC chicken sperm during storage.

In contrast, 0.9% NaCl solution showed a lower storage period for viability, which was 72 hours (2±4%). This is due to the cold shock that occurs when using NaCl diluent, causing sperm clumping during storage. Consequently, this diluent results in lower sperm survival compared to Ringer lactate. Cold shock during sperm storage triggers stress on the sperm membrane through free radicals, causing damage (Sanocka and Kurpisz 2004; Thuwanut et al. 2011). Meanwhile, PBS solution maintains viability up to 120 hours (0.5±1.9%), higher than 0.9% NaCl solution but lower than RL. This is because PBS has a higher capacity than NaCl to maintain the solution's pH, likely due to its non-toxic buffering properties for spermatozoa. According to Martin et al. (2006), PBS is isotonic and non-toxic to cells, with the ability to maintain osmolarity.

Fig. 6 shows that the viability of KBC spermatozoa at observation times 0h, 12h, 24h, and 36h showed no significant difference ($P>0.05$) between the use of 0.9% NaCl diluent, RL, and PBS at 0h and 12h. However, at 24h and 36h, a decrease in viability was observed, resulting in a significant difference ($P<0.05$) between RL and NaCl and PBS solutions. There was no significant difference between PBS and NaCl solutions. Observations of KBC spermatozoa viability during storage (0h, 12h, 24h, and 36h) were based on semen quality (viability of KBC spermatozoa) in the range (≥40%).

Storage at low temperatures slows down the metabolic process, thus slowing the production of free radicals from metabolism. Examination of spermatozoa viability is carried out by staining with eosin-negrosin. Sperm that suffer plasma membrane damage lose their semi-permeability. The plasma membrane cannot select the entry and exit of substances due to osmotic pressure, so when the eosin-negrosin color test is conducted, the eosin enters the plasma, turning the spermatozoa red. This indicates that the spermatozoa have died due to increased cell membrane permeability (Gacem et al. 2021).

To evaluate the viability of Kokok Balenggek chicken spermatozoa in commercial physiological solutions stored at 4ºC, various studies on the effects of different additives and diluents on sperm quality and viability offer valuable insights. Diba et al. (2023) demonstrated that adding egg yolk to a saline solution extender enhanced the motility and viability of rooster spermatozoa at cool temperatures. This suggests that incorporating egg yolk into physiological solutions could improve the viability of Kokok Balenggek chicken spermatozoa during storage at 4ºC. Furthermore, Khaeruddin et al. (2024) found that including Butylated Hydroxytoluene (BHT) in the diluent increased the motility, viability, plasma membrane integrity, and acrosome integrity of chicken spermatozoa. This indicates that incorporating specific antioxidants like BHT could be advantageous for maintaining the quality of Kokok Balenggek chicken spermatozoa in physiological solutions. Moreover, Rakha et al. (2016) emphasized that diluting

chicken spermatozoa with specific extenders, such as the Beltsville Poultry semen extender, led to better sperm plasma membrane integrity during storage. This suggests that selecting the appropriate extender is crucial in preserving the viability of Kokok Balenggek chicken spermatozoa at 4ºC. Based on the referenced studies, it can be inferred that including egg yolk, antioxidants like BHT, and selecting suitable extenders could potentially enhance the viability and quality of Kokok Balenggek chicken spermatozoa when stored in commercial physiological solutions at 4ºC.

Conclusion

The study demonstrated that Ringer's Lactate (RL) is the most effective solution for preserving the motility and viability of Kokok Balenggek rooster spermatozoa at 4ºC, outperforming both 0.9% NaCl and Phosphate Buffered Saline (PBS). Spermatozoa stored in RL maintained higher motility and viability over an extended period, indicating its superior preservation capability. These findings suggest that RL can significantly enhance poultry reproductive management and semen storage practices, offering a reliable method to improve artificial insemination outcomes and overall breeding efficiency in the poultry industry.

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Author's contribution

All authors contributed significantly to the research and manuscript preparation. Ananda was responsible for conceptualization, methodology, data curation, and writing the original draft. Jaswandi contributed to formal analysis, investigation, and reviewing and editing the manuscript. Rusfidra provided resources, supervision, validation, and project administration. Raziah Sri Wahyuni handled visualization, software, data analysis, and also contributed to reviewing and editing the manuscript.

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