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Original article

Spermatozoa motility and viability of turkey (*Meleagris gallopavo*) semen in egg yolk nanoparticles-phosphate buffered saline extender at 3-5°C storage

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ABSTRACT

This study aimed to evaluate the spermatozoa motility and viability of turkey semen extended with egg yolk nanoparticles-PBS extender at various concentrations, stored at 3-5°C. Semen was collected from two turkeys and divided into four treatment groups. Group T0 consisted of fresh turkey semen extended with 20% (v/v) fresh egg yolk in phosphate-buffered saline (PBS). Groups T1, T2, and T3 consisted of fresh turkey semen extended with 5, 10, and 20% (v/v) egg yolk nanoparticles in PBS, respectively. Extended semen was stored in a refrigerator (3-5°C), and progressive spermatozoa motility and viability were evaluated every two hours until spermatozoa motility decreased to 40%. The results showed that turkey semen in T3 extender containing 20% egg yolk nanoparticles in PBS was able to maintain spermatozoa motility for up to eight hours, better than T0 and T2 extenders which supported spermatozoa motility for six hours. In contrast, T1 extenders only support spermatozoa motility for four hours. T3 extenders were also able to maintain spermatozoa viability for up to eight hours, while T0, T1 and T2 extenders could only support viability for 6 hours. The extender with 20% egg yolk nanoparticles in PBS was significantly better ($p < 0.05$) in maintaining spermatozoa quality compared to those with 5 and 10% egg yolk nanoparticles, as well as the extender with 20% fresh egg yolk. In conclusion, 20% egg yolk nanoparticles in PBS semen extender was effective in maintaining the motility and viability of turkey (*Meleagris gallopavo*) spermatozoa stored at 3-5°C for up to eight hours.

Keywords: chilled storage, motility, phosphate buffered saline, spermatozoa quality, viability

INTRODUCTION

Turkey husbandry has experienced significant advancements in recent years, driven by increasing demand for alternative sources of animal protein and increasing awareness of the nutritional benefits associated with turkey meat (Hafez and Shehata, 2021). The increasing demand for poultry products, particularly turkey, was primarily due to shifts in consumer preferences toward healthier dietary options. Turkey meat was known for its high protein content (crude protein 14.1-16.5%), and low-fat levels 1.01-2.68% (Solaesa *et al*., 2024), making it particularly appealing to health-conscious consumers. This increasing demand was

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encouraging farmers and entrepreneurs to invest in turkey farming as a viable business opportunity to meet the needs of this market (van Staaveren *et al*., 2020). The future of turkey farming looked promising, with great potential for expansion and diversification. As consumer awareness regarding healthy eating increased, along with increasing interest in turkey as a premium meat choice, producers had a unique opportunity to expand their supply and penetrate domestic and export markets (Hossain *et al*., 2024). Additionally, research and development initiatives focused on breeding efficiency, feed optimization, and disease resistance would be crucial to ensuring the competitiveness and sustainability of the turkey farming sector (Hafez and Shehata, 2021).

The adoption of advanced farming technologies had played a crucial role in enhancing turkey production efficiency. Innovations in breeding techniques, such as artificial insemination (AI), had effectively addressed the natural breeding challenges associated with size discrepancies between male and female turkeys (Baskota *et al*., 2023). In poultry, AI could be performed using fresh semen, cold-stored semen, and frozen semen (Donoghue and Wishart, 2000); however, frozen semen has not yet been widely utilized in turkey breeding practices. The advantages of using cold-stored semen compared to fresh semen for AI in turkeys are significant. By cooling semen to temperatures around 4°C, the metabolic activity of spermatozoa was reduced, thereby extending their viability to several days. This extended storage capability offered increased flexibility in the timing of AI procedures, particularly in coordinating breeding schedules (Suwimonteerabutr *et al*., 2024).

The use of chilled semen facilitated more efficient breeding management by allowing producers to collect semen from genetically superior males and distributed it to multiple females without the need for immediate insemination (Tvrdá *et al*., 2023). This practice was particularly advantageous in commercial operations, where synchronizing breeding times could enhance conception rates and optimize genetic advancements within the flock (Wang *et al*., 2024). Furthermore, utilizing chilled semen for AI provided several additional benefits compared to fresh semen. These benefits included reduced stress on breeding males, increased genetic diversity, advantages in disease management, cost-effectiveness, and opportunities for standardization and quality control. Collectively, these factors contributed to the efficiency and efficacy of turkey breeding programs, ultimately supporting advancements in poultry genetics and productivity (Mohan et al., 2018). Effective semen storage at cold temperatures necessitated the use of an appropriate extender. An effective extender had to meet several criteria, including providing essential nutrients for spermatozoa, being non-toxic to spermatozoa, and protecting against cold shock (Bustani and Baiee, 2021). A decline in spermatozoa quality during storage could occur due to disruptions in the permeability of the spermatozoa cell membrane, which resulted from changes in the liquid environmental conditions caused by secretions from the male genital glands prior to adding the extender (Suwimonteerabutr *et al*., 2024). Furthermore, spermatozoa metabolism continued even when liquid semen was stored at low temperatures; this metabolic process required energy and leads to the depletion of energy sources during prolonged storage periods (Kheawkanha *et al*., 2023).

Common extenders for poultry semen included egg yolk phosphate extenders. Egg yolk is rich in lipoproteins and lecithin, which played a crucial role in protecting the spermatozoa cell membrane by encapsulating it (Bustani and Baiee, 2021). Fresh egg yolk particles were macromolecules measuring approximately 14.46 ± 0.33 µm. The effectiveness of egg yolk in safeguarding the spermatozoa membrane could be enhanced by reducing the size of the egg yolk particles to the nanoscale. Nanoparticles measuring less than 1000 nm (Ariantie, 2020). To date, the use of egg yolk nanoparticles for the preservation of turkey semen has not been documented. Therefore, this study aimed to evaluate the viability and motility of turkey spermatozoa preserved in an egg yolk

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nanoparticles extender compared to traditional egg yolk phosphate at a storage temperature of 3-5°C.

MATERIALS AND METHODS

Semen used in this research was obtained from two adult male turkeys (*Meleagris gallopavo*).

Extender preparation

Phosphate Buffer Saline (PBS) was prepared by dissolving one phosphate buffer tablet in 100 mL of distilled water, followed by heating the solution on an electric stove for 10 minutes while stirring until it is homogeneous (Situmorang *et al*., 2014).

Preparation of egg yolk nanoparticles-phosphate buffered saline extender

The process of producing egg yolk nanoparticles was carried out using a milling method, in which a grinding chamber was employed to break the egg yolk particles into smaller sizes. The eggs to be used were first cleaned their shell with 70% alcohol, then cracked open, followed by separating the yolk from the egg white. The intact egg yolk, still surrounded by its membrane, was rolled on filter paper to remove any residual egg white. The egg yolk membrane was then broken, and the yolk was transferred into a measuring cylinder until a volume of 50 mL was reached. This yolk was then mixed with 400 mL of distilled water. The mixture was placed into the grinding chamber, which was operated at a speed of 11,600 rpm and at a temperature range of -10°C to -15°C. The milling process lasted for 110 minutes, following a cycle of 1-minute milling and 4-minute pause. Particle size analysis of the egg yolk after milling was performed using Dynamic Light Scattering (DLS) to ensure that the particle size was below 1 µm (Ariantie, 2020). Finally, egg yolk nanoparticles were mixed with PBS and the antibiotics Penicillin and Streptomycin, respectively at concentrations of 1000 IU and 1000 µg per mL of extender until the mixture was homogeneous (Situmorang *et al*., 2014).

Semen collection

The collection of turkey semen was carried out using the milking method, which began with cleaning the cloacal area with a cotton swab moistened with physiological saline (NaCl). This procedure involved two individuals, one person held the turkey while the other milked and collected the semen (Kucera *et al*., 2018). The process was conducted continuously until the male's papilla becomes visible at the cloaca. Following this, the area around the cloaca was massaged until semen was released, at which point the ejaculation reflex was subsided. The expelled semen was then collected in a pre-cleaned container to ensure protection against contamination (Girndt *et al*., 2017).

Fresh semen evaluation

Examination of the collected semen was carried out macroscopically and microscopically. Macroscopic assessment included the evaluation of volume, color, odor, and pH, and consistency. The volume was determined by referring to the graduation on the semen collection tube, while the color was observed by examining the semen in a transparent collection tube under bright light. Odor was assessed through the sense of smell, pH was measured using a pH indicator paper, and consistency was assessed by tilting the tube containing the semen and then returning it to its original position to observe any residue adhering to the walls of the collection tube (Ramahtia *et al*., 2024).

Microscopic examination of semen included assessing concentration, observing mass and individual motility, as well as determining the percentages of spermatozoa motility and viability. The concentration of spermatozoa was estimated using the Russian method, where a drop of semen was placed on a microscope slide, covered with a cover slip, and examined under a microscope at 400x magnification. This allowed observing the distance between the heads of individual spermatozoa which were categorized into several classifications. Semen was classified as densum (D) if the distance between spermatozoa heads was less than the length of

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one spermatozoa head (indicating more than 1,000,000 spermatozoa per mm³ of semen); semi-densum (SD) if the distance was greater than the length of one spermatozoa head (contains between 500,000 to 1,000,000 spermatozoa per mm³ of semen); rarum (R) if the distance was approximately equal to the total length of one spermatozoa (with a number of spermatozoa less than 500,000); and azoospermia (A) if no spermatozoa or only a few spermatozoa were found in the semen (Gitayana *et al*., 2023).

The observation of spermatozoa mass motility was carried out by placing a drop of semen on a microscope slide and examining it under a magnification of 100x. Spermatozoa mass motility was categorized into several groups namely excellent (+++) if the observed wave motion was large, thick, abundant, and moved quickly; good (++) if the waves were thin, sparse, and tended to be slow; moderate (+) when thjere were no visible mass wave of spermatozoa, and motile spermatozoa tend to move individually; and poor (0) or undetectable (N) when no movement was observed at all. The mass motility categories that qualify for AI or preservation were those rated (+++) and (++) (Ramahtia *et al*., 2024).

Dilution procedure

Fresh semen that met quality standards was diluted in an extender in a ratio of one-part fresh semen to ten parts of extender. The four extenders used was T0, which was a PBS extender containing 20% (v/v) fresh egg yolk, and T1, T2, and T3, which were PBS extender respectively containing 5, 10, and 20% (v/v) egg yolk nanoparticles. All extenders were supplemented with antibiotics to achieve concentrations of 1000 IU/mL of Penicillin and 1000 µg/mL of Streptomycin (Gitayana *et al*., 2023). Each semen sample in the extenders was then stored in a refrigerator at 5°C and evaluated every hour for a duration of eight hours.

Motility assessment

The assessment of the percentage of progressive motility of spermatozoa was carried out by mixing semen and physiological saline (NaCl). The mixture was then placed on a microscope slide and covered with a cover slip. Motility was observed under a microscope with 400x magnification to calculate the percentage of spermatozoa that showed progressive movement. Observations were carried out by counting spermatozoa in several fields of view, with a minimum number of 100 spermatozoa assessed (Gitayana *et al*., 2023).

Viability assessment

The determination of spermatozoa viability was performed through eosin-nigrosin staining. A drop of extended semen was placed on a microscope slide and mixed with eosin-nigrosin stain until homogeneous. Examination was then carried out using a microscope at a magnification of 400x. Dead spermatozoa absorb the dye due to the weakening or damage of their cell membranes. The percentage of spermatozoa viability was calculated by observing multiple fields of view, with a minimum of 100 spermatozoa counted (Gitayana *et al*., 2023).

Data Analysis

Differences in motility and viability between the two treatments are presented in tabular form and analyzed using Analysis of Variance followed by Duncan's Multiple Range Test. All data were processed using SPSS version 23.

RESULTS

The results of the fresh semen examination conducted prior to the treatments are presented in Table 1.

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Table 1 Macroscopic and microscopic parameters of fresh turkey semen

macroscopic parameters		microscopic parameters	
volume (mL)	0.48 ± 0.04	concentration	densum
color	milky white	viability (%)	87.40 ± 2.79
odor	specific	mass movement	+++
consistency	thick	individual movement	progressive
pH	7	motility (%)	79.00 ± 6.52

The highest average percentage of motility was found in the T3, T0, and T2 treatment groups, while the lowest percentage of motility was observed in the T1 treatment group. At 0 hour of storage there was no significant difference ($p > 0.05$) between the control group and all treatment groups. At 2 hours of storage, there was a significant difference ($p < 0.05$) between T1 and T3; however, T1 did not show significant differences ($p > 0.05$) when compared to T0 and T2. Furthermore, T0 and T2 were not significantly differ ($p > 0.05$) from T3. At 4 hours and 6 hours of storage, T0, T1, and T2 did not show significant differences ($p > 0.05$), but were significantly different ($p < 0.05$) from T3. At the 8 hours, T3 was significantly different ($p < 0.05$) from T0, T1, and T2 (Table 2).

At 0 hour of storage, treatment T0 (control group) and all other treatments did not show significant differences ($p > 0.05$). At 2 hours of storage, T0 was also not significantly different ($p > 0.05$) from other treatments; however, T1 was significantly different ($p < 0.05$) from T3. At 4, 6, and 8 hours of storage, T3 showed a significant difference ($p < 0.05$) compared to T0, T1, and T2. Throughout the storage period of 0 to 8 hours, the highest percentage of viability was found in T3, while the lowest percentage was found in T1 (Table 3).

Tabel 2 Motility of turkey spermatozoa in extenders containing egg yolk nanoparticles

	0 hour	2 hours	4 hours	6 hours	8 hours
T0	72.00 ± 4.47 ^{a}	62.00 ± 2.73 ^{ab}	51.00 ± 4.18 ^{a}	42.00 ± 2.73 ^{a}	33.00 ± 2.73 ^{b}
T1	69.00 ± 6.51 ^{a}	58.00 ± 6.70 ^{a}	49.00 ± 5.47 ^{a}	39.00 ± 2.23 ^{a}	29.00 ± 2.23 ^{a}
T2	71.00 ± 6.51 ^{a}	62.00 ± 5.70 ^{ab}	52.00 ± 2.73 ^{a}	41.00 ± 2.23 ^{a}	33.00 ± 2.73 ^{b}
T3	75.00 ± 5.00 ^{a}	66.00 ± 5.47 ^{b}	59.00 ± 4.18 ^{b}	51.00 ± 2.23 ^{b}	42.00 ± 2.73 ^{c}

T0: fresh turkey semen extended in phosphate-buffered saline (PBS) containing 20% (v/v) fresh egg yolk; T1, T2, and T3: fresh turkey semen extended in PBS containing 5, 10, and 20% (v/v) egg yolk nanoparticles, respectively. Different superscripts in the same column indicate a significant difference ($p < 0.05$).

Tabel 3 Viability of turkey spermatozoa in extenders containing egg yolk nanoparticles

	0 hour	2 hours	4 hours	6 hours	8 hours
T0	79.80 ± 4.27 ^{a}	69.40 ± 4.93 ^{ab}	58.40 ± 4.88 ^{a}	46.80 ± 3.83 ^{a}	37.40 ± 3.36 ^{ab}
T1	76.80 ± 4.76 ^{a}	64.20 ± 3.83 ^{a}	54.80 ± 3.27 ^{a}	44.00 ± 4.30 ^{a}	33.20 ± 3.42 ^{a}
T2	79.20 ± 4.03 ^{a}	68.80 ± 4.76 ^{ab}	59.20 ± 4.55 ^{a}	47.80 ± 3.70 ^{a}	38.40 ± 4.39 ^{b}
T3	80.60 ± 2.70 ^{a}	72.60 ± 2.07 ^{b}	64.60 ± 2.79 ^{b}	57.60 ± 2.19 ^{b}	47.60 ± 2.07 ^{c}

T0: fresh turkey semen extended in phosphate-buffered saline (PBS) containing 20% (v/v) fresh egg yolk; T1, T2, and T3: fresh turkey semen extended in PBS containing 5, 10, and 20% (v/v) egg yolk nanoparticles, respectively. Different superscripts in the same column indicate a significant difference ($p < 0.05$).

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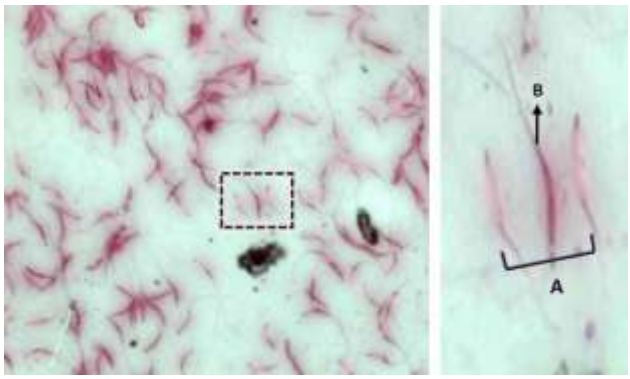

Figure 1 Spermatozoa viability assessed by eosin nigrosine staining; live spermatozoa are characterized by heads that appear white (A), while dead spermatozoa are characterized by heads that appear purplish (B); viewed under a light microscope at 400x magnification.

DISCUSSION

The volume of semen produced by poultry during each ejaculation could vary, influenced by several factors, including health status, condition of reproductive organs, and frequency of collection (Pimprasert *et al*., 2023). Semen ejaculated by two male turkeys in five collection sessions yielded a good volume ranging from 0.4 to 0.5 mL. The odor observed in the semen studied was characteristic of turkey semen, presenting a fishy odor reminiscent of eggs, accompanied by a slight sulfur aroma. Normal turkey semen typically possessed a distinctive fishy odor, which reflected the animal itself (Alkan *et al*., 2002). The color and consistency of semen could be an indicator to quickly predict the concentration of spermatozoa in a sample. Semen which was milky white in color and had a thick consistency was expected to contain a high concentration of spermatozoa. The semen collected in this study displayed a milky white color with a thick consistency, as evidenced by the residue adhering to the walls of the container after tilting. Quality assessment of the turkey semen also indicated a normal pH of 7, with values typically ranging from 7.0 to 7.6.

Microscopic examination revealed a motility percentage ranging from 72% to 89%, indicating the presence of a substantial number of progressive spermatozoa. High motility enabled spermatozoa to reach the ova in the fallopian tubes more quickly, thereby facilitating fertilization (Ichikawa *et al*., 2016; López-Pérez *et al*., 2021). The assessment of fresh spermatozoa motility included evaluations of both mass motility and individual motility, where high-quality semen was characterized by an abundance of progressive spermatozoa and a minimum mass motility rating of (++). The evaluation of semen motility from five collection sessions showed promising results, with mass motility categorized as (+++) and individual motility indicating a significant presence of progressive spermatozoa. Spermatozoa viability was assessed using eosin-nigrosin staining, which differentiated between live and dead spermatozoa; live spermatozoa remained unstained, while dead spermatozoa were stained reddish-purple. The percentage of viability observed in the fresh turkey spermatozoa within this study ranged from 84% to 92%.

Spermatozoa motility

The storage of liquid semen at low temperatures from the 0 hour to the 8 hours indicated that the average percentage of motility in spermatozoa was highest in T3, followed by T0, T2, with the lowest percentage observed in T1. Liquid semen deemed suitable for insemination was defined as having at least 40% motile spermatozoa. Treatment T3 was able to maintain its quality for 8 hours, exceeding the quality retention of semen in treatments T0 and T2 by 2 hours. In contrast, the semen in treatment T1 was barely able to retain its quality for 6 hours under low-temperature storage. The motility in treatment T3 demonstrated the best results compared to the other three treatments. The addition of 20% egg yolk nanoparticles to semen extender was found to maintain spermatozoa motility more effectively than those contained 5% and 10% egg yolk nanoparticles, as well as 20% fresh egg yolk. Therefore, using 20% egg yolk in the extender for turkey semen is recommended. The combination of this

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recommended dosage of egg yolk and the smaller size of the egg yolk particles enveloping the spermatozoa may provide additional protection for the spermatozoa.

This experiment demonstrated that the average percentages of motility for treatments T2 and T0 during 0 to 8 hours storage period exhibited no significant differences. This finding indicates that the use of 20% fresh egg yolk and 10% egg yolk nanoparticles were equally effective in preserving spermatozoa motility during 6 hours of low-temperature storage. This effect may be attributed to the small size of the egg yolk nanoparticles, which was considered more effective at enveloping the spermatozoa cell membrane, thereby maintaining spermatozoa motility (Najafi *et al*., 2022), comparably to the 20% fresh egg yolk extender, which was the recommended dosage for diluting turkey semen. The size of the egg yolk nanoparticles was measured at 381.21 ± 6.21 nm, while the fresh egg yolk measured 14,460.00 ± 330.76 nm. The small size of the extender particles enhanced their interaction with the spermatozoa cell membrane, and optimizing protective functions. The use of nanomaterials was likely to yield favorable outcomes, even when smaller amounts were used compared to larger particle materials (Sun *et al*., 2021). Phosphate-buffered saline is a non-toxic buffer solution for spermatozoa, acting as a buffering fluid to prevent shock during pH changes (Martin *et al*., 2006).

The decrease in spermatozoa motility during storage can be attributed to several factors, including changes in the environmental conditions of the liquid resulting from the secretions of male reproductive glands. When these secretions mix with the extender solution, they may induce stress that is detrimental to spermatozoa motility (Janosikova *et al*., 2023). This adaptation process disrupts the permeability of the spermatozoa membranes. Furthermore, spermatozoa metabolism continued even when liquid semen was stored at low temperatures, which required energy for metabolic activities (Paventi *et al*., 2022). The duration of liquid semen storage affected the decline in spermatozoa motility due to the depletion of nutrients that serve as energy sources over time (Rochmi and Sofyan, 2019).

Spermatozoa viability

From hour 0 to hour 8 of liquid semen storage at low temperatures, the average percentage of spermatozoa viability was highest in treatment T3, followed by T2, T0, and the lowest percentage was observed in T1. This indicates that the addition of 20% egg yolk nanoparticles in the semen extender was the most effective compared to 5% and 10% egg yolk nanoparticles, as well as 20% fresh egg yolk. The percentage of egg yolk added to the semen extender significantly affected the durability of liquid semen quality (Chankitisakul *et al*., 2022).

The addition of 20% egg yolk nanoparticles was better maintained semen quality due to its sufficient dosage for nourishing spermatozoa and the beneficial interaction between the nanoparticles and the spermatozoa cell membrane, allowing for more thorough encapsulation of the spermatozoa surface. The optimal content of phospholipids in semen extender could be achieved through the addition of 20% egg yolk. This concentration was more effective than the addition of 10% egg yolk in preserving spermatozoa quality during storage. Nanoparticles exhibited superior interaction capabilities with target cells compared to macroparticles and microparticles. This enhanced effectiveness was attributed to the increased surface area of the extender particles that enveloped the spermatozoa cell membranes, thereby effectively preserving spermatozoa viability during storage (Orzołek *et al*., 2021). Conversely, the addition of 5% egg yolk nanoparticles to turkey semen extenders was less effective than the other three percentages, likely due to an insufficient quantity to maintain the quality of liquid semen.

The spermatozoa cell membrane consisted of fatty acids and cholesterol, which were essential for maintaining membrane function and integrity (Janosikova *et al*., 2023). The viability of spermatozoa during storage could be preserved by adding egg yolk to the semen

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extender, as egg yolk extenders contained low-density lipoproteins, proteins, fatty acids, cholesterol, and phospholipids. These components played a crucial role in protecting the spermatozoa cell membrane during preservation. The constituents of egg yolk could encapsulate the surface of the spermatozoa cell membrane and simultaneously helped replace components that might have been lost during processing (Bergeron *et al*., 2004). The preservation of liquid semen at low temperatures was essential for extending storage duration (Partyka and Niżański, 2022). Turkey semen stored at low temperatures could maintain its quality for 6 to 12 hours. The cooling process, transitioning from room temperature to lower temperatures, has the potential to induce shock, which could lead to damage to the spermatozoa cell membrane and resulted in spermatozoa cell mortality (Vasicek *et al*., 2015). Although the metabolic processes of spermatozoa in semen stored at low temperatures continued, they occurred at a reduced rate. This prolonged the duration of storage, which impacted spermatozoa viability, resulting in a continual decline in the percentage of viable spermatozoa (Chankitisakul *et al*., 2022). Additionally, the metabolism of spermatozoa produced lactic acid, and its accumulation could decrease the pH of the liquid semen, potentially becoming toxic to the spermatozoa (Matsuzaki *et al*., 2015). Damage to the membrane could lead to disturbances in permeability, significantly increasing cell permeability and allowing eosin stain to enter the cells. This was indicated by changes in the color of the spermatozoa head; dead spermatozoa exhibited a reddish-purple coloration (Gacem *et al*., 2021).

This study indicates that spermatozoa motility and viability of turkey semen extended in phosphate-buffered saline containing 20% egg yolk nanoparticles were higher compared to those extended in phosphate-buffered saline containing 20% fresh egg yolk, 10% egg yolk nanoparticles, or 5% egg yolk nanoparticles.

CONCLUSION

A dose of 20% egg yolk nanoparticles in phosphate buffered saline semen extender was most suitable for maintaining the motility and viability of turkeys (*Meleagris gallopavo*) spermatozoa during storage at 3-5°C for eight hours.

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AUTHOR'S CONTRIBUTIONS

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NAS conceived the idea and designed the main framework of this manuscript, as well as handled data acquisition, analysis, and interpretation, and drafted the manuscript under the supervision of HAH and EPH. TWS, and TH critically reviewed and revised the manuscript for intellectual content. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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