Supplementation of Glycine and Glucose into Egg Yolk Lactated Ringer Diluent on The Quality of Local Chicken Semen Stored at 5ºC for 120 Hours

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Abstract

The impact of supplementing glucose, glycine, or a combination of both in Ringer's lactate egg yolk base extender to preserve the quality of semen from local Indonesian chickens has not been previously investigated. This study aimed to examine the potential of glucose and glycine on chicken semen stored at 5°C for 120 hours. In this study, five local roosters were used. The parameters under observation included semen volume, odor, pH levels, consistency, color, mass movement, concentration, motility, viability, abnormality, plasma membrane integrity, chromatin degeneration, and acrosomal cap integrity. This study was conducted using a completely randomized design (CRD) with four treatments groups and 10 replication, i.e. T1 (control without supplementation), T2 (50 mM glucose), T3 (60 mM glycine), and T4 (a combination of 50 mM glucose and 60 mM glycine), respectively. In result, semen volume was 0.54 ± 0.17 mL/ejaculate, a milky white color, distinctive odor, thick consistency, good mass movement (++/+++), pH of 7.37 ± 0.23, motility of 91.50 ± 2.42%, plasma membrane integrity of 96.85 ± 0.96%, abnormality at 2.88 ± 0.77%, the concentration of 3.04 ± 0.3 billion/mL, and viability of 96.47 ± 1.71%. Following storage at 5°C for 120 hours, the motility, viability, abnormality, and acrosomal cap integrity of local chicken spermatozoa significantly different ($p < 0.05$) between T3 and T4 compared to T1 and T2 groups. Moreover, the integrity of the plasma membrane and chromatin degeneration in treatment T3 significantly different ($p < 0.05$) from T1, T2, and T4 groups. In conclusion, local chickens exhibited fair quality fresh semen both in macroscopic and microscopic evaluations. Furthermore, the combination of 60 mM glycine and 50 mM glucose into local chicken semen stored at 5°C for 120 hours effectively preserved motility and viability, minimized abnormality, maintained plasma membrane integrity, minimized chromatin degeneration, and retained acrosomal integrity.

Keywords: glucose, glycine, local chicken, semen

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INTRODUCTION

Indonesia boasts a diverse array of local chicken breeds, showcasing significant variations in phenotypic appearance, production performance, growth, and reproduction characteristics (Hernawati and Safitri, 2020). With approximately 34 registered types of local chickens (Henuk and Bakti, 2018), there is a pressing need to develop and enhance their genetic quality and population. The advancement of local chicken breeds with superior genetic potential relies heavily on implementing an effective mating system (Fikri and Purnama, 2020). The prevalent natural mating system in local chicken rearing poses challenges, including a relatively high risk of inbreeding, which can adversely impact chicken production performance. KUB chicken is a local poultry breed developed at the Livestock Research Center (BALITNAK), aiming to enhance both egg and meat production. BALITNAK, an arm of the Agricultural Research and Development Agency dedicated to producing superior agricultural products, has actively engaged in research and development resulting in the creation of high-performing local livestock breeds. One such success story is KUB chickens, celebrated for its superior egg and meat production capabilities. Artificial insemination (AI) emerged as a pivotal reproductive technology applicable to chicken breeding. The success of AI in chickens

significantly depends on the quality of the inseminated semen (Hastuti *et al*., 2021). Semen quality is contingent upon storage conditions, notably influenced by both storage temperature and the choice of extender (Wurlina *et al*., 2020).

Spermatozoa from the rooster's reproductive organs require specific handling to minimize quality deterioration. Semen, containing spermatozoa, needs to be mixed with the extender to increase volume and uphold quality when stored at low temperatures. Storing semen in cold temperatures serves as a solution to the limited durability of fresh semen at room temperature (Andaruisworo *et al*., 2023). The objective of semen storage is to slow spermatozoa metabolism, preserving their viability and fertility (Heydari *et al*., 2021). The motility and fertilizing capacity of spermatozoa in fresh poultry semen decline within an hour after collection (Dumpala *et al*., 2006; Hernawati and Safitri, 2020). Short-term storage of poultry semen at 4°C necessitates appropriate extenders to sustain spermatozoa viability (Getachew, 2016). Brillard (2009) and Susilowati *et al*. (2018) highlighted the importance of storing semen at low temperatures, typically ranging from 4–10°C.

Carbohydrates are commonly incorporated into semen extenders. Besides serving as a cryoprotectant, carbohydrates act as an essential energy source for spermatozoa (Yendraliza *et al*., 2023). Chicken semen contains relatively fewer carbohydrates compared to mammalian semen (Garner and Hafez, 2000), necessitating the addition of carbohydrates to the semen extender. Glucose is the primary carbohydrate found in chicken seminal plasma (Li *et al*., 2010). However, the glucose concentration in bird seminal plasma is typically very low and, in some cases, undetectable (Azzam *et al*., 2022). Glucose plays a crucial role as an energy source for spermatozoa, produced through the glycolysis process, which is essential for sperm motility (Setiawan *et al*., 2020).

The spermatozoa plasma membrane, abundant in unsaturated fatty acids, is highly susceptible to damage through lipid peroxidation. Free radicals, highly reactive entities, can readily interact with these unsaturated fatty acids, initiating attacks by liberating hydrogen atoms and forming free lipid radicals that further react with lipid peroxides. During storage, spermatozoa are prone to free radical attacks (oxidation), leading to potential spermatozoa degeneration (Wajdi *et al*., 2021). Antioxidants in semen are known to decrease during dilution and storage phases (Kutluyer and Kocabas, 2016). Therefore, storing semen necessitates the addition of antioxidant components capable of combating free radicals to prevent spermatozoa degeneration due to oxidative stress. Amino acids have demonstrated antioxidant properties (Atessahin *et al*., 2008). Glycine, an amino acid with antioxidant capabilities, can be introduced into the semen extender. Studies have shown that the inclusion of glycine in the extender effectively maintains the quality of bull and buffalo spermatozoa (El-Sheshtawy *et al*., 2008). Additionally, study by Moreno and Lucero (2019) supports the notion that specific amino acids uphold the viability percentage and DNA integrity of chicken spermatozoa.

The supplementation of glucose, glycine, and their combination in Ringer's lactate base extender for maintaining the quality of local Indonesian chicken semen has not been previously explored. This study aimed to assess the potential of glucose and glycine for chicken spermatozoa during storage at 5°C for 120 hours.

MATERIALS AND METHODS

Ethical Approval

The procedures, mechanisms, and entire series of study underwent evaluation and approval by the Animal Care and Use Committee of Brawijaya University, Malang, East Java, Indonesia, on June 19, 2023, under certificate number 056-KEP-UB-2023.

Study Period and Location

The semen was collected from local KUB chickens, which are a product developed at the Livestock Research Center (BALITNAK). The rearing of these local chickens took place at the Livestock Coop of the Agricultural Development Polytechnic (POLBANGTAN) of Malang. The

preparation of the extender, testing of fresh semen, and assessment of semen quality post-storage were conducted at the Health and Reproduction Laboratory of the Agricultural Development Polytechnic of Malang.

Animals

In this study, five local roosters were used as samples. Each chicken was housed in individual cages measuring 40 × 50 × 70 cm and provided a complete feed of 200 g/day, along with *ad libitum* access to drinking water.

Semen Extender Preparation

The basic extender comprised 90% Ringer's lactate solution and 10% egg yolk. The egg yolk was dissolved in Ringer's lactate and then centrifuged at 3000 rpm for 10 minutes, with the resulting supernatant serving as the fundamental extender. To this supernatant, 1000 IU/mL penicillin and 1 mg/mL streptomycin were added, and the pH was adjusted to 7.4 using Tris hydroxymethyl aminomethane. The preparation of the extender was performed according to specific treatments i.e. (T1) control, (T2) 50 mM glucose, (T3) 60 mM glycine, and (T4) the combination of 50 mM glucose and 60 mM glycine. Subsequently, the extender was distributed into four tubes, each containing a different treatment, and homogenized. The composition of the extender is detailed in Table 1.

Table 1. Composition of treatment diluent materials

Composition	Treatment			
	T1	**T2**	**T3**	**T4**
RLEY (mL)	10	10	10	10
Glucose (mM)	0	50 mM	0	50 mM
Glycine (mM)	0	0	60 mM	60 mM
Penicillin (IU/mL)	1000	1000	1000	1000
Streptomycin (mg/mL)	1	1	1	1

RLEY= 90% Ringer's lactate supernatant and 10% egg yolk; 50 mM= 0.09 g of glucose, 60 mM= 0.045 g of glycine.

Collection and Evaluation of Fresh Semen

The semen was collected using the massage method and subjected to both macroscopic and microscopic evaluations. The macroscopic assessment included volume, odor, pH, consistency, and color, while the microscopic evaluation involved assessing motility, viability, abnormalities, plasma membrane integrity, and spermatozoa concentration using microscope (Olympus CX41, Japan).

Semen evaluation for motility was conducted at 0, 24, 48, 72, 96, and 120 hours of storage. The assessment of viability, abnormality, plasma membrane integrity, acrosomal cap integrity, and chromatin degeneration was performed at 0, 72, and 120 hours of storage.

Macroscopic Assessments

Macroscopic assessments included observations on volume, odor, pH, consistency, and color. Semen volume was quantified using a graduated tube, while pH measurement employed specialized indicator paper. Semen consistency was categorized as thick, medium, or thin, and its color was distinguished between cream, milky white, and clear. Additionally, the scent of the semen was categorized as distinctive, fishy, or pungent.

Mass Sperm Motility

Fresh semen was carefully dripped onto a glass surface and observed using a light microscope at 100× magnification. The evaluation considered the thickness of the mass wave and the speed of movement across the field. The criteria for assessment were categorized as follows: very good (+++/3) indicating fast and thick mass sperm motility, good (++/2) for slow but thick mass sperm motility, fair (+/1) denoting slow and thin mass sperm motility, and poor (0) indicating no observable waves (Junaedi *et al*., 2016).

Spermatozoa Motility

To observe spermatozoa motility, 10 µL of diluted semen was carefully dripped onto a warm (37°C) object glass, covered with a cover glass, and assessed using a light microscope at 400× magnification. The observation technique involved scanning in a zig-zag pattern across the object glass to randomize the field of view. Progressive motility was calculated by distinguishing actively progressive spermatozoa from those moving in place, backward, in circles, or remaining static. Observations were conducted across a minimum of five to ten fields of view (Junaedi *et al.*, 2016).

Viability

Eosin-nigrosine dye was used to assess spermatozoa viability. The examination was performed under a light microscope at 400× magnification across ten fields of view (Junaedi *et al.*, 2016).

Abnormality

Spermatozoa abnormality was examined using eosin-nigrosine dye to distinguish between normal and abnormal morphology. The examination was conducted using a microscope at 400× magnification. Spermatozoa abnormality was calculated based on a minimum count of 200 spermatozoa or assessed across 10 fields of view. The percentage of abnormal spermatozoa was categorized into primary and secondary abnormalities (Alkan *et al*., 2002).

Spermatozoa Concentration

The spermatozoa concentration was determined as the number of spermatozoa per milliliter using a Neubauer counting chamber with a 500 times dilution (998 µL 3% NaCl with 2 µL semen). Diluted semen was evenly distributed on both sides of the counting chamber glass. Observations were made using a light microscope at 100× magnification. The total spermatozoa from five Neubauer counting chambers, averaged across both sides, were then multiplied by 25×10^6 (considering the dilution factor, correction from counting 5 counting boxes, and conversion from mm^3 to mL). The formula used for calculating spermatozoa concentration was spermatozoa concentration (spermatozoa/mL) = the average number of spermatozoa on both sides of the Neubauer counting chamber (Junaedi *et al*., 2017).

Plasma Membrane Integrity

Plasma membrane integrity was determined using the hypoosmotic swelling test. For this, 10 µL of semen was diluted in 100 µL of a hypoosmotic solution prepared by dissolving 0.09 g of fructose and 0.049 g of sodium citrate in 10 mL of distilled water. The diluted solution was then incubated at 37°C for 30 minutes. Next, the semen was carefully applied onto a pre-warmed at 45°C glass slide and covered with a cover glass (Mehdipour *et al*., 2016; Najafi *et al.*, 2021).

A total of 200 spermatozoa or 10 fields of view were observed under a light microscope at 400× magnification. Identification of spermatozoa with intact plasma membranes was based on criteria proposed by Santiago-Moreno *et al*. (2012), which include characteristics such as a bent tail, folded tip of the tail, bent-middle, shortened, and thickened tail. Spermatozoa with incomplete plasma membranes were recognized by a straight tail due to the absence of a membrane reaction to the solution.

Chromatin degeneration

A total of 10 µL semen sample was added to a prepared slide and allowed to dry. The slides were then fixed in a solution of 96% ethanol and acetone (1:1) at 4°C for 30 minutes before air-drying at room temperature for an additional 30 minutes. Subsequently, the slides underwent hydrolysis in 0.1 N HCl at 4°C for 5 minutes, followed by rinsing with distilled water three times, each for 2 minutes, and then air-dried at room temperature. Toluidine blue was applied to the prepared slides and left at room temperature for 20 minutes. Afterward, distilled water was used to rinse the slides, followed by air drying at room temperature (Rui *et al*., 2017). The observations were conducted using a light microscope at 400× magnification, assessing a minimum of 10 fields of view. Identification of

chromatin degeneration involved evaluating spermatozoa heads for chromatin integrity. Spermatozoa with intact chromatin displayed a bright blue or clear appearance, whereas those with defective chromatin integrity appeared dark blue or purple. The dye bound to phosphate groups in DNA strands, indicating poor chromatin density. Spermatozoa with good chromatin density showed little or no coloring by the toluidine blue dye. Examination was performed on a minimum of 100 spermatozoa per sample (Kim *et al*., 2013).

Acrosome Cap Integrity

A total of 10 µL semen drop was placed on a slide, followed by the addition of 10 µL of 5% formalin. The mixture was homogenized and spread evenly across the slide, then allowed to dry. The preparations were fixed in 5% formalin at 37°C for 30 minutes and rinsed thoroughly with distilled water. Subsequently, they were immersed in Coomassie brilliant blue – R 250 solution for 5 minutes, rinsed again with distilled water, and dried. The preparations were observed under a microscope at 1000× magnification, assessing a minimum of 10 fields of view (Silyukova *et al*., 2022).

Data Analysis

SPSS 25.0 was used for statistical analysis. Inferential analysis was conducted to examine mean differences among all treatments. Data with a normal distribution underwent comparative statistical tests using One-Way ANOVA at a significance level (α) of 0.05. Post hoc tests were performed if the data indicated significance at $p < 0.05$.

RESULTS AND DISCUSSION

Quality of Local Chicken Fresh Semen

The quality of fresh semen from local chickens for macroscopic and microscopic observations were reported in Table 2. The volume of local chicken semen produced in this study was measured at 0.54 ± 0.17 mL/ejaculate. Notably, the semen volume of the local chickens in this study exceeded that of previous studies: 0.24 mL (Hambu *et al*., 2016), 0.39 mL (Dos Santos *et al*., 2018), and 0.38 mL (Du *et al*., 2016). The quantity of ejaculated spermatozoa is influenced by the endocrine system, primarily regulated by the pituitary gland and testicles (Getachew, 2016; Rahma *et al*., 2021). Spermatozoa production is initiated by the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, leading to the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior lobe of the pituitary. Gonadal steroids are subsequently secreted (Guo *et al*., 2015). LH stimulates the Leydig cells in the testicles to produce progesterone, which is then converted into the hormone testosterone (Saputro *et al*., 2022). Testosterone is crucial for the development of secondary sex characteristics, normal mating behavior in male livestock, functioning of the accessory glands, spermatozoa production, and maintenance of the male duct system. Moreover, testosterone aids in transporting spermatozoa and semen deposition within the female reproductive tract (Prayogo *et al*., 2022).

The semen of local chickens typically exhibits a milky white color, a distinctive odor, and a thick consistency. A milky white appearance and thick consistency indicate high spermatozoa concentration and robust motility, whereas a clear, watery white color signifies lower spermatozoa concentration. The mass spermatozoa motility in chickens is graded as good (++/+++), with noticeable large and swift-moving waves. Mass spermatozoa motility is closely associated with individual sperm motility, as noted by Hambu *et al*. (2016), suggesting that mass movement reflects individual motility. The pH level recorded in this study was 7.37 ± 0.23, considered within the normal range for local chicken semen. This aligns with Shoyebo *et al*. (2008), that chicken semen typically exhibits a pH between 6.0 and 8.0. pH levels significantly impact motility (Yang *et al*., 2019), wherein lower pH can reduce spermatozoa movement, subsequently reducing sperm viability (Sarkar, 2020).

Table 2. Quality of fresh semen from local chickens

Parameters	Mean ± SD
Volume (ml)/ejaculation	0.54 ± 0.17
Color	Milky white
Odor	Distinctive smell
pH	7.37 ± 0.23
Consistency	Thick
Sperm concentration (x 10^6/ml)	3035 ± 303.6
Mass spermatozoa motility (+)	++/+++
Spermatozoa motility (%)	91.50 ± 2.42
Spermatozoa viability (%)	96.47 ± 1.71
Plasma membrane integrity (%)	96.85 ± 0.96
Abnormality morphology	2.88 ± 0.77

Table 3. Percentage of spermatozoa mortality in local chicken semen stored at 5ºC

Storage Time (Hours)	Treatment			
	T1	T2	T3	T4
0	91.00 ± 3.16^a	91.50 ± 5.30^a	90.50 ± 3.69^a	91.50 ± 3.37^a
24	59.50 ± 5.50^a	63.00 ± 5.87^b	67.50 ± 4.25^b	68.00 ± 4.22^b
48	45.50 ± 4.97^a	55.00 ± 5.77^b	60.00 ± 5.27^c	59.00 ± 4.59^{bc}
72	42.00 ± 6.32^a	46.50 ± 4.12^b	54.50 ± 6.43^c	51.50 ± 5.80^{bc}
96	33.50 ± 4.12^a	40.50 ± 4.38^b	46.50 ± 5.80^c	42.50 ± 5.89^{bc}
120	30.00 ± 6.24^a	34.50 ± 3.69^b	38.00 ± 4.22^b	36.00 ± 6.15^b

Different superscripts in the same row showed significant differences ($p < 0.05$).

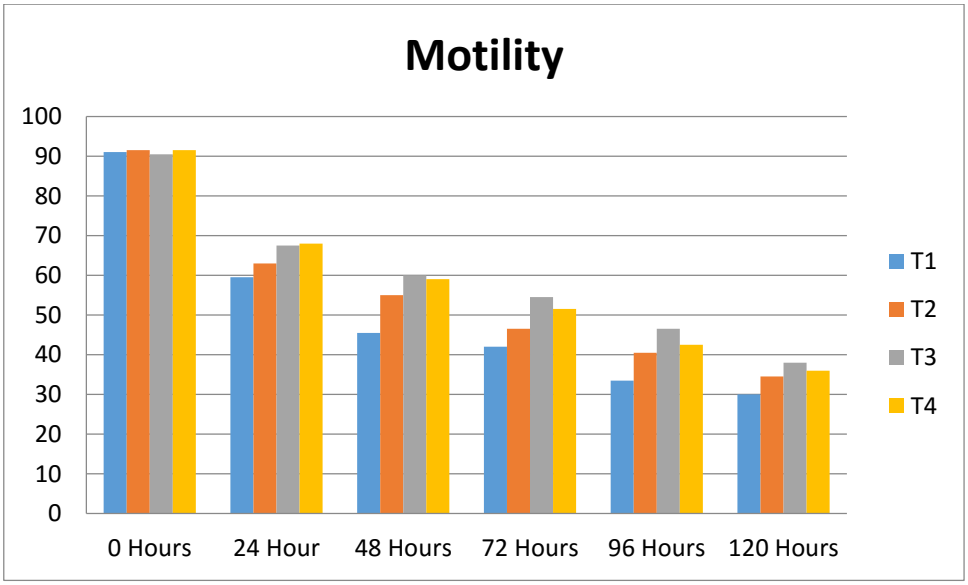

Figure 1. Trend of decreasing motility of stored spermatozoa from 0 to 120 hours.

Table 4. Percentage of spermatozoa viability in local chicken semen stored at 5ºC

Treatment	Stroge Time (Hours)		
	0	72	120
T1	97.81 ± 0.38^a	64.97 ± 2.49^a	57.82 ± 3.45^a
T2	98.25 ± 0.09^b	76.95 ± 3.99^b	69.44 ± 5.28^b
T3	98.31 ± 0.33^b	80.34 ± 1.59^c	74.45 ± 3.36^c
T4	98.14 ± 0.46^b	80.91 ± 2.23^c	74.46 ± 1.75^c

Different superscripts in the same column showed significant differences ($p < 0.05$).

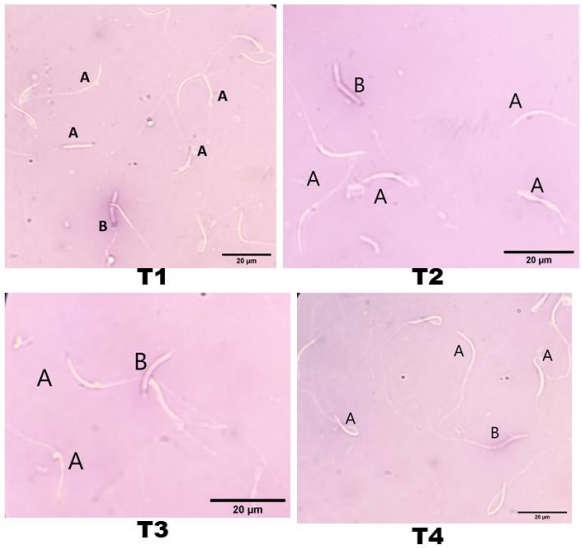

Figure 2. (A) Life spermatozoa (do not absorb color); (B) Dead spermatozoa (absorb color).

Table 5. Spermatozoa abnormality in local chicken semen stored at 5ºC

Treatment	Storage Time (Hours)		
	0	72	120
T1	2.32 ± 0.35[a]	10.40 ± 0.60[a]	15.36 ± 1.11[a]
T2	2.73 ± 0.43[a]	8.12 ± 1.47[b]	10.99 ± 0.86[b]
T3	2.90 ± 0.95[a]	6.32 ± 0.94[c]	10.82 ± 2.37[b]
T4	3.04 ± 0.88[a]	6.44 ± 0.78[c]	9.87 ± 0.82[b]

Different superscripts in the same column showed significant differences ($p < 0.05$).

Table 6. Plasma membrane integrity of spermatozoa in local chicken semen stored at 5ºC

Treatment	Storage Time (Hours)		
	0	72	120
T1	97.72 ± 0.54[a]	86.76 ± 4.41[a]	69.74 ± 1.73[a]
T2	96.55 ± 1.20[b]	82.33 ± 3.21[a]	69.77 ± 1.89[a]
T3	96.59 ± 1.04[b]	87.87 ± 1.56[b]	76.24 ± 2.27[b]
T4	96.54 ± 0.61[b]	82.74 ± 4.08[b]	69.53 ± 3.89[a]

Different superscripts in the same column showed significant differences ($p < 0.05$).

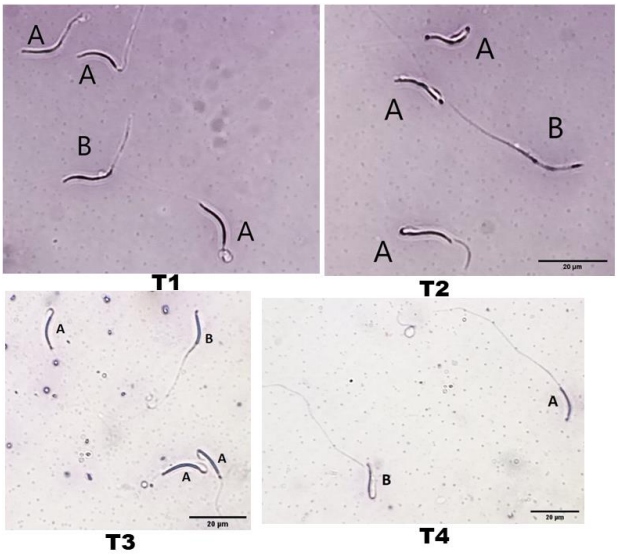

Figure 3. (A) Plasma membrane integrity; (B) Degeneration of plasma membrane.

Table 7. Chromatin degeneration of spermatozoa in local chicken semen stored at 5ºC

Treatment	Storage Time (Hours)		
	0	72	120
T1	1.36 ± 0.31[a]	3.35 ± 0.71[a]	5.62 ± 1.16[a]
T2	1.21 ± 0.46[a]	2.93 ± 0.71[a]	4.52 ± 0.75[b]
T3	1.01 ± 0.19[a]	2.38 ± 0.49[b]	3.41 ± 0.57[c]
T4	1.14 ± 0.36[a]	2.84 ± 0.19[ab]	4.21 ± 0.62[b]

Different superscripts in the same column showed significant differences ($p < 0.05$).

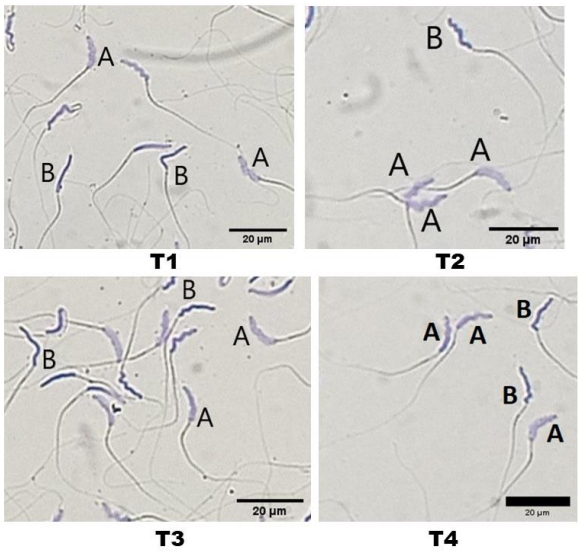

Figure 4. (A) Chromatin integrity; (B) Chromatin degeneration.

Table 8. Integrity of acrosomal cap of spermatozoa in local chicken semen stored at 5ºC

Treatment	Storage Time (Hours)		
	0	72	120
T1	98.98 ± 0.37[a]	94.33 ± 1.13[a]	90.96 ± 0.75[a]
T2	98.43 ± 0.35[a]	94.48 ± 1.35[a]	90.48 ± 0.59[a]
T3	99.16 ± 0.22[a]	95.27 ± 0.59[a]	92.65 ± 1.15[b]
T4	98.17 ± 0.17[a]	95.29 ± 1.47[a]	91.50 ± 2.20[ab]

Different superscripts in the same column showed significant differences ($p < 0.05$).

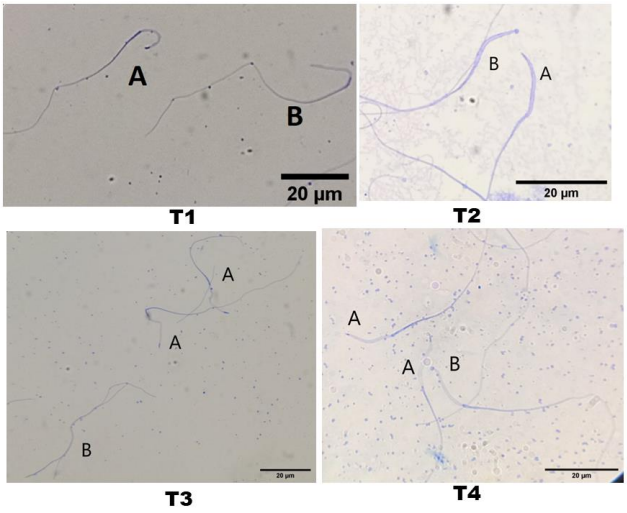

Figure 5. (A) Integrity of acrosomal cap; (B) Disintegrity of acrosomal cap.

Microscopic evaluation of local chicken spermatozoa quality revealed motility at 91.50 ± 2.42%, plasma membrane integrity at 96.85 ± 0.96%, and abnormality at 2.88 ± 0.77%. Motility serves as a crucial parameter for assessing semen quality and predicting fertility in animals (Chatiza *et al*., 2012). The concentration of local chicken spermatozoa was notably high at 3.04 ± 0.3 billion/mL. Spermatozoa concentration plays a pivotal role in determining the appropriate dosage for artificial insemination. Additionally, the observed spermatozoa viability at 96.47 ± 1.71% is considered good, as indicated by Lukman (2014), specifying that viable spermatozoa typically fall within the range of 60–75% (Saputro *et al*., 2020). The results from testing the quality of fresh semen from local chickens indicate that the spermatozoa possess characteristics meeting the quality criteria for processing and artificial insemination (AI) procedures (Junaedi *et al*., 2023).

Spermatozoa Motility

The motility of local chicken spermatozoa after storage at 5℃ for 120 hours in all treatment groups were reported in Table 3. The results indicated significant differences ($p < 0.05$) between T2, T3, and T4 treatments compared to T1 across the storage period from 24 to 120 hours. Treatment T3 maintained motility above 45% for up to 96 hours of storage compared to others (Figure 1). Specifically, semen stored for 96 hours with 60 mM glycine added to Ringer's lactate-egg yolk extender showed significant improvement ($p < 0.05$) compared to other extenders. Glycine, an amino acid functioning as an antioxidant, plays a crucial role in preserving spermatozoa quality during storage. Antioxidants act to shield spermatozoa from degeneration caused by free radicals and lipid peroxidation. This aligns with findings from Khaeruddin *et al*. (2020) and Iswati *et al*. (2017), emphasizing that adding antioxidants to chicken semen preserves spermatozoa quality. Spermatozoa motility significantly impacts rooster fertility, essential for reaching the oocyte (Morrell, 2019). Assessing motility stands as a vital measure of semen quality for artificial insemination (AI); thus, chicken spermatozoa with over 45% motility are deemed suitable for AI purposes.

Spermatozoa motility in semen typically decreases over the storage period, aligning with findings by Blank *et al*. (2021), which note a significant decline in spermatozoa motility post-storage. Semen storage induces changes in spermatozoa quality due to increased oxidative stress, which in turn diminishes motility (Víquez *et al*., 2020). Low motility in chicken spermatozoa is often attributed to lipid peroxidation (Mussa *et al*., 2020), as chicken spermatozoa, rich in polyunsaturated fatty acids (Breque *et al*., 2003), are particularly susceptible to this process (Cerolini *et al*., 2006). Moreover, reduced motility during storage can be linked to increased movement and metabolic activity. The motility of spermatozoa demands energy, yet spermatozoa have limited energy production capability outside the body (Purnama *et al*., 2019). Glucose, a simple carbohydrate, serves as an essential energy source for spermatozoa. It undergoes metabolism to generate adenosine triphosphate (ATP), crucial for the movement of spermatozoa tails. Setiawan *et al*. (2020) study highlighted glucose's role in ATP production through glycolysis and oxidative phosphorylation, vital for flagella movement and fertility in chicken spermatozoa.

Spermatozoa Viability

The viability of local chicken spermatozoa after storage at 5℃ was reported in Table 4. The analysis revealed significant differences ($p < 0.05$) in spermatozoa viability between treatments T3 and T4 compared to T1 and T2 groups. Glycine, acting as an antioxidant, and glucose, as an energy source, contribute to maintaining spermatozoa viability. Antioxidants naturally present in semen diminish over storage time, enhancing viability in semen diluent supplemented with glycine compared to those without. During storage, semen quality can diminish due to intracellular ice crystal formation (Hezavehei *et al*., 2018) and inhibited by radical oxygen species (ROS) (Zhang *et al*., 2021). Glycine functions as an antioxidant, counteracting free radicals and minimizing the

production of hydrogen peroxide and superoxide anions, thereby curbing reactive oxygen species (ROS) formation and reducing lipid peroxidation (Dewi *et al*., 2023). Consequently, this minimizes plasma membrane degeneration caused by free radicals (Hanifah *et al*., 2020).

The viability test in Figure 2 uses the eosin-negrosin staining technique which gives dead spermatozoa a red color because the membrane is not functioning properly, so the dye enters the spermatozoa. Spermatozoa that are not colored indicate that the spermatozoa are still alive because the membrane is still functioning properly so that the dye does not enter the spermatozoa (Hidayatik *et al*., 2021). Degeneration to the spermatozoa membrane, especially in the acrosome, is detected during viability assessments (Safitri *et al*., 2022). Viability stands as a crucial benchmark for estimating the lifespan of spermatozoa within the female reproductive tract, thereby serving as an indicator of spermatozoa quality (Sukmawati *et al*., 2014).

Spermatozoa Abnormality

Abnormalities of local chicken spermatozoa after storage at 5℃ are shown in Table 5. The analysis of local chicken spermatozoa abnormality at 5°C storage revealed significant differences ($p < 0.05$) between treatments T3 and T4 compared to T1 and T2 groups. Treatments T3 and T4 groups were effective in minimizing abnormality for up to 120 hours. Spermatozoa abnormality refers to structural or physical changes in spermatozoa that deviate from normal conditions. Such abnormalities can impact the spermatozoa's ability to move or fertilize eggs, thereby affecting fertilization success. There are various morphologies of spermatozoa abnormalities, with the most common being secondary abnormalities characterized by broken, curved, coiled, and shortened tails. Degeneration to the tail of spermatozoa hampers their movement, consequently affecting their motility (Alkan *et al*., 2002).

Spermatozoa Plasma Membrane Integrity

The integrity of the plasma membrane of local chicken spermatozoa when storing semen at 5ºC for 120 hours was reported in Table 6. Observations on the plasma membrane integrity values of local chicken spermatozoa revealed significant differences ($p < 0.05$) between treatments T2, T3, and T4 groups. The Ringer's lactate-egg yolk base extender supplemented with 60 mM glycine proved highly effective in maintaining plasma membrane integrity, showcasing a smaller reduction level compared to others (Figure 3). In chickens, the plasma membrane of spermatozoa serves as a semipermeable barrier, susceptible to osmotic shock from abrupt changes in osmotic pressure, resulting in membrane degeneration. Storage at low temperatures induces cold shock, altering the phospholipids composing the spermatozoa membrane. This cold shock during semen storage triggers stress on the spermatozoa membrane through free radicals, causing spermatozoa degeneration (Sanocka and Kupisz, 2004; Thuwanut *et al*., 2011). The low-temperature storage induces cold shock by loosening the phospholipid bonds within the spermatozoa membrane (Susilowati *et al*., 2019; Utomo and Safitri, 2018; Purnama *et al*., 2019).

The integrity of spermatozoa plasma membranes was assessed through the hypoosmotic swelling test to determine their condition. Spermatozoa with intact membranes typically display bent tails, folded tail tips, bent middle parts, shortened tails, and thickened tails, whereas degeneration membranes result in straight tails. The plasma membrane serves as a crucial protective layer in spermatozoa, pivotal for the fertilization process. Over time, the percentage of plasma membrane integrity in local chicken spermatozoa declined due to the semen storage process, influenced by temperature and osmolarity changes, prompting the generation of ROS or free radicals (Nebel, 2007). These free radicals induce lipid peroxidation, potentially causing substantial degeneration to the spermatozoa plasma membrane's carbon covalent bonds within its phospholipid bilayer (Gogol and Wierzchos-Hilczer, 2009; Ogbuewu *et al*., 2010).

Antioxidants, compounds that counteract free radicals, play a role in reducing lipid degeneration. Glycine, known for its antioxidant properties, aids in shielding spermatozoa from oxidative stress-induced degeneration (Wijayanti *et al*., 2023). By integrating glycine into semen diluents, the risk of spermatozoa structural degeneration, including that to the plasma membrane, can be mitigated.

Spermatozoa Chromatin Degeneration

Observations of chromatin degeneration to semen stored for up to 120 hours can be seen in Table 7. The analysis of spermatozoa chromatin degeneration among the four treatments revealed significant differences ($p < 0.05$) during semen storage. Treatment T3 demonstrated the capacity to minimize spermatozoa chromatin degeneration compared to other treatments, yielding notably lower percentages: 2.38 ± 0.49% at 72 hours of storage and 3.41 ± 0.57% at 120 hours of storage. The quality of chromatin within the cell nucleus is pivotal, tightly bound to protamine as a DNA protector. Chromatin quality in sperm is contingent upon the composition of protamine and histones. Mature sperm typically contain at least 85% protamine and 15% histones (Carrell *et al*., 2007). A higher ratio of protamine to histones facilitates denser packaging of sperm DNA. Failures in the transformation of histones into protamine and suboptimal chromatin density are linked to infertility issues (Hekmatdoost *et al*., 2009). Chromatin maturity and density in spermatozoa are crucial for tightly packaging the paternal genome, shielding it from mutagens and potential DNA degeneration. The chromatin density of spermatozoa also contributes to enhancing the nucleus' hydrodynamics, aiding spermatozoa in quicker movement toward the ovum for fertilization (Oliva R, 2006).

Examining spermatozoa chromatin degeneration is crucial for the initial diagnosis of male fertility (Erenpreisa *et al*., 2003). For successful fertilization, spermatozoa require intact chromatin (Morrell and Rodriguez-Martinez, 2009). Degeneration to spermatozoa chromatin can occur during spermatogenesis or semen storage due to several factors, such as hormones and high levels of ROS (Cheng *et al*., 2015). When chromatin is degenerated, it can lead to delays in nuclear fusion during fertilization and result in embryo death (Cordova *et al*., 2002). Protamine, the primary protein in the spermatozoa nucleus, plays a crucial role in forming chromatin and binding to DNA (Arpanahi *et al*., 2009). This protein is essential for the proper structure of spermatozoa chromatin. The abnormal expression of protamine leads to increased chromatin degeneration in spermatozoa. During spermatogenesis, particularly the spermiogenesis stage, approximately 85% of the spermatozoa nuclei in the form of histones are replaced by protamine (Wykes and Krawetz, 2003). This replacement of histones by protamine is vital for the maturation of spermatozoa (Arpanahi *et al*., 2009).

Toluidine blue staining is a technique used to evaluate spermatozoa chromatin density. This method operates on the principle that the toluidine blue dye binds to phosphate groups found in spermatozoa DNA strands exhibiting poor chromatin density (Figure 4). Spermatozoa with good chromatin density show minimal or no staining when exposed to toluidine blue dye (Tsarev *et al*., 2009). In this process, the acrosome of spermatozoa with good chromatin density appears bright blue or clear, while that of spermatozoa with poor chromatin density appears purple or dark blue. Changes in chromatin directly impact DNA status. The degree of chromatin condensation influences the resulting color intensity, reflecting the quality of this process when employing toluidine blue staining. A more compact and intact condensation yields a bright blue color, whereas less compact or incomplete condensation appears dark blue. Non-compact condensation is associated with chromatin abnormalities and compromises spermatozoa chromatin integrity (Erenpreiss *et al*., 2001).

Spermatozoa Acrosome Cap Integrity

The integrity of the acrosomal cap of local chicken spermatozoa when storing semen at 5ºC for 120 hours was reported in Table 8. The acrosomal cap of spermatozoa in treatments T3

and T4 showed significant differences ($p < 0.05$) compared to treatments T1 and T2 groups. There was a notable decrease in intact acrosomal caps from the beginning to the end of semen storage. With prolonged storage, spermatozoa tend to undergo an increased acrosome reaction, leading to more instances of spermatozoa lacking an intact acrosomal cap. The acrosomal cap, found on the head of spermatozoa, serves to shield the release of genetic material and enzymes (Figure 5). The acrosome possesses an elongated cone shape, with its tip containing enzymes crucial for spermatozoa to penetrate the oocyte. Its vesicle comprises proteolytic enzymes (Ahammad *et al*., 2013) that, upon an acrosome reaction, are released by spermatozoa. These enzymes facilitate contact with the perivitelline layer during the fertilization process in the oocyte (Blesbois, 2018).

Storing semen can impede the ability of the acrosome reaction, thus affecting the fertility of rooster spermatozoa. The acrosomal hood plays a pivotal role in successful fertilization during mating. Its function in the fertilization process is closely linked to the enzymes it contains. Degeneration to the acrosomal cap results in the release of enzymes, leading to a loss of spermatozoa's ability to fertilize (Nguyen *et al*., 2015).

CONCLUSION

The combination of 60 mM glycine and 50 mM glucose into local chicken semen stored at 5°C for 120 hours effectively preserved motility and viability, minimized abnormality, maintained plasma membrane integrity, minimized chromatin degeneration, and retained acrosomal integrity.

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AUTHORS' CONTRIBUTIONS

TS: Conceptualization and drafted the manuscript. MHN and TS: Validation, supervision, and formal analysis. J, MHN, and NI: Performed sample evaluation. J and TS: Performed the statistical analysis and the preparation of tables and figures. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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