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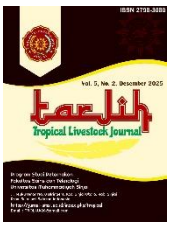

Motility and Viability of Kampung Chicken Sperm Using Diluents Containing Glycine and Glucose

La Ode Muhammad Aswad Salam[1], La Ode Muhammad Iskandar Zulkarnain[1], Rani Rahmadani[1], Junaedi[1*], Khaeruddin[2]

[1]Department of Animal Husbandry, Faculty of Agriculture, Fisheries and Animal Husbandry, Universitas Sembilanbelas November Kolaka. Jl. Pemuda No. 339, Kolaka 93517, Southeast Sulawesi, Indonesia.
[2]Program of Animal Science, Faculty of Agriculture, Universitas Muhammadiyah Sinjai.
Jl. Teuku Umar, No. 8, 92611, North Sinjai, South Sulawesi, Indonesia.
*junaedi.peternakan@gmail.com

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ABSTRACT

The success of an artificial insemination program in native chickens is largely determined by the quality of liquid semen used during storage. This study aimed to evaluate the effect of diluents containing 30 mM glucose, 30 mM glycine, and a combination of both on the motility and viability of native chicken liquid semen stored at 3-5°C. The study used a Completely Randomized Design (CRD) with four treatments: control, 30 mM glucose, 30 mM glycine, and a combination of 30 mM glucose-glycine, each with five replications. Fresh semen from three native roosters aged 10-14 months was collected using the massage method, then diluted with a Ringer's lactate-egg yolk-based diluent at a ratio of 1:5. Evaluation of spermatozoa motility and viability was carried out daily for three days of storage. Data were analyzed using ANOVA followed by Duncan's Multiple Range Test at a significance level of 5%. The results showed that on the first day, all treatments had motility 75.76-75.85% and viability 87.03-88.32% which were not significantly different (P>0.05). On the second and third days, the 30 mM glycine treatment and combination showed significantly higher motility 42.02-42.20% and 33.19-33.22% respectively, and viability 67.87-67.92% and 57.89-57.90% respectively compared to the control and single glucose. The 30 mM glucose treatment did not show any superiority over the control. It was concluded that 30 mM glycine supplementation, either alone or in combination with glucose, effectively maintained the quality of native chicken liquid semen for up to three days of storage through osmoprotection and antioxidative mechanisms.

INTRODUCTION

Indonesian local chickens are a type of poultry germplasm with high genetic potential and have long been cultivated by the community as an important source of animal protein. The local chicken population in Indonesia reaches over 300 million, spread across various regions with relatively high genetic diversity (Nataamijaya, 2010). The

advantages of local chickens include good adaptation to tropical environments, disease resistance, and the production of meat and eggs with a distinctive flavor favored by local consumers. However, the productivity of local chickens remains relatively low compared to purebred chickens, necessitating efforts to improve genetic quality through planned and sustainable breeding programs (Junaedi *et al*., 2016a). One reproductive technology that can be applied to accelerate the genetic improvement of local chickens is artificial insemination (AI), which allows for optimal and efficient use of superior males (Junaedi *et al*., 2021).

Artificial insemination in poultry is a rapidly developing technology and has been proven to increase reproductive efficiency and accelerate the dissemination of superior genetics from superior males to a wider population. According to Kharayat *et al*. (2016) and Widyasanti *et al.* (2025), the use of artificial insemination (AI) in poultry can increase fertility, reduce the male-to-female ratio, prevent the spread of sexually transmitted diseases, and facilitate more targeted selection and crossbreeding programs. The success of an AI program is largely determined by the quality of the semen used, which includes parameters such as motility, viability, concentration, and morphology of spermatozoa. Freshly ejaculated semen has optimal quality, but cannot always be used immediately due to distance and time constraints between the semen collection location and the insemination location. Therefore, semen preservation technology is needed that can maintain spermatozoa quality during storage, both in liquid and frozen semen forms (Abouelezz *et al*., 2015).

Semen diluents are crucial components in semen preservation technology, protecting spermatozoa from damage caused by cold shock, providing nutrients for sperm cell metabolism, maintaining appropriate pH and osmotic pressure, and inhibiting the growth of microorganisms (Junaedi *et al*., 2017). Various types of diluents have been developed for the preservation of chicken semen, ranging from simple diluents to complex diluents containing various components such as egg yolk, milk, phosphate buffer, and antibiotics (Khairuddin et al., 2019; Khaeruddin *et al.*, 2020; Sedani *et al.,* 2025; Rakha et al., 2024). Glucose is one of the most widely used diluent components because it acts as the main energy source for spermatozoa to maintain their motility and viability during storage. Research by Khaeruddin *et al.* (2024) showed that the addition of glucose to diluents can increase the durability of chicken spermatozoa during storage at low temperatures (5 °C). Another study by Khaeruddin & Kurniawan (2020) also examined the use of glucose in frozen chicken semen. On the other hand, glycine, a non-essential amino acid, is known to have a protective effect on spermatozoa cell membranes from damage due to oxidative stress (Neamah, 2022; Said *et al*., 2019). The combination of glucose as an energy source and glycine as a protective agent is expected to synergistically maintain the quality of liquid semen from local chickens during storage.

Sperm motility is one of the most important parameters in evaluating semen quality because it directly correlates with fertilization ability. Sperm must have good progressive motility to be able to reach the sperm storage tubule (Sayed *et al*., 2023), and then exit towards the yolk, penetrating the perivitelline layer of the egg for fertilization (Nishio & Matsuda, 2017). Decreased motility during semen storage can be caused by various factors such as depletion of energy reserves, accumulation of toxic metabolic products, plasma membrane damage, and oxidative stress due to excessive production of reactive oxygen species (ROS). In addition to motility, spermatozoa viability, which reflects the percentage of viable sperm cells, is also an important indicator of semen quality. Viability can be assessed based on the integrity of the spermatozoa's plasma membrane, as a damaged plasma membrane indicates cell death or irreversible damage (Moce & Graham, 2008).

Maintaining spermatozoa motility and viability at optimal levels during liquid semen storage is a major challenge in AI applications in local chickens, considering that Indonesia's relatively hot tropical environmental conditions can accelerate semen quality deterioration.

This study aims to evaluate the effect of diluents containing glucose and glycine at various concentrations on the motility and viability of liquid semen from local chickens stored at 3-5°C. The information obtained from this study is expected to contribute to the development of an effective and economical diluent formula for the preservation of local chicken semen, thereby supporting the success of artificial insemination programs and improving the genetic quality of local chickens in Indonesia. In addition, this study is also expected to provide a deeper understanding of the protective mechanisms of glucose and glycine on chicken spermatozoa during cold storage, which ultimately can be used as a basis for the development of more advanced poultry reproductive technology in the future. Thus, efforts to increase productivity and preserve the germplasm of Indonesian local chickens can be carried out more efficiently and sustainably through the application of appropriate reproductive technology.

METHODS

Experimental Animals

The research material used was fresh semen from adult local male chickens meeting the following criteria: 10-14 months of age, 1.8-2.2 kg body weight, healthy and free of physical defects, and trained for semen collection. Three roosters were used, housed individually in 40 x 50 x 45 cm battery cages. The chickens were fed a commercial feed with a crude protein content of 16-18% and a metabolizable energy of 2,800-2,900 kcal/kg, at a rate of 100 grams per chicken per day. Drinking water was provided ad libitum. Lighting was provided for 12 hours per day to maintain optimal reproductive performance. The chickens were acclimatized to the semen collection procedure for two weeks prior to the study to reduce stress and ensure consistent semen quality.

Materials

The materials used in this study include: analytical-grade glucose ($C_6H_{12}O_6$), analytical-grade glycine ($C_2H_5NO_2$), 1000 IU/ml penicillin and 1000 µg/ml streptomycin to prevent bacterial contamination, fresh chicken egg yolk as a protective component for spermatozoa membranes, Ringer's lactate as a solvent, and eosin-nigrosin solution for viability staining.

Design

This is a laboratory experimental study using a Completely Randomized Design (CRD), with treatments of lactated ringer diluent-egg yolk (control), lactated ringer diluent-egg yolk supplemented with 30 mM glucose, lactated ringer diluent-egg yolk supplemented with 30 mM glycine, and lactated ringer diluent-egg yolk supplemented with a combination of glucose and glycine, with each treatment repeated five times. This study used repeated measurements to assess the quality of semen stored on the first and second days.The study used repeated measures to assess the quality of semen stored on the first and second days.

Procedure

Diluent Preparation

The basic diluent was prepared with the following composition: 90% Ringer's lactate, 10% egg yolk, 1000 IU/ml penicillin, and 1000 µg/ml streptomycin. Glucose and glycine were added according to the treatment concentrations. Each diluent combination was mixed homogeneously using a magnetic stirrer for 15 minutes at room temperature. The pH of the diluent was measured and adjusted to 6.8-7.2 using a pH meter. The finished diluent was then stored in a refrigerator at 3-5°C for three days.

Semen Collection

Semen collection was carried out in the morning between 7:00 and 9:00 a.m. WIB using a massage method. The rooster to be collected was removed from the cage and held in a proper manner to avoid stress. Massage was performed from the back towards the tail and around the cloaca with gentle movements until ejaculation occurred. The released semen was immediately collected in a collection tube. Only good quality semen was used in the study, with an individual motility criterion of at least 70%.

Semen Dilution and Storage

Semen meeting quality criteria from all roosters was mixed (pooled semen) to standardize quality and reduce individual variation. The semen was then diluted with each treatment diluent at a ratio of 1:5 (1 semen: 5 diluents). The dilution process was carried out slowly at room temperature to avoid cold shock. The diluted semen was then transferred to test tubes labeled according to the treatment, at a volume of 0.25 ml per tube. The tubes containing the liquid semen were then stored in a refrigerator at 3-5°C. Storage continued until day 3, with semen quality evaluations conducted daily.

Semen Quality Evaluation

Sperm motility was evaluated by taking a 10 µl semen sample using a micropipette and dropping it onto a glass slide that had been warmed to 37°C on a hot plate. The sample was covered with a coverslip and immediately observed under a microscope at 400x magnification. Progressive sperm motility was observed in at least five different fields of view, and the percentage of progressively moving spermatozoa was calculated (Junaedi *et al*., 2016b).

Sperm viability was evaluated using the eosin-nigrosin staining method. A 10 µl semen sample was mixed with eosin-nigrosin solution on a glass slide, then a thin smear was made and allowed to air-dry. The smear was observed under a microscope at 400x magnification. Live spermatozoa did not absorb the dye (remained white/clear), while dead spermatozoa absorbed the red color from the eosin. Counts were performed on at least 200 spermatozoa in several different fields of view.

Data Analysis

Data obtained from observations of sperm motility and viability were analyzed using analysis of variance (ANOVA) with an F-test at a 5% significance level ($\alpha = 0.05$) to determine the effect of the treatment on the observed variables. If the analysis of variance results indicated a significant ($P<0.05$) or highly significant ($P<0.01$) effect, further testing was performed using Duncan's Multiple Range Test (DMRT) to determine differences between treatments.

All statistical analyses were performed using statistical software such as SPSS version 25 with a 95% confidence level.

RESULT AND DISCUSSION

Sperm Motility

The results showed that all treatments had relatively similar sperm motility values on the first day of storage (75.76-75.85%), indicating that the addition of glucose, glycine, or a combination of the two did not negatively impact the initial quality of liquid semen. This indicates that the diluent used had good compatibility with native chicken spermatozoa. High initial motility (>70%) is an important indicator of a successful semen dilution process, as it reflects the ability of spermatozoa to maintain their viability immediately after collection and dilution (Rakha *et al*., 2021). Consistent initial motility values across all treatments also confirmed that semen handling protocols had been implemented effectively, so any differences that appeared the following day were purely a result of the treatment.

Table 1. Sperm motility of Kampung chickens using different liquid semen treatments during storage

Treatments	Motility (%)		
	Day 1	Day 2	Day 3
Control	75.80±6.76[a]	41.37±5.34[a]	32.13±4.44[ab]
Glucose 30 mM	75.76±8.67[a]	41.39±9.67[a]	31.26±7.66[b]
Glycine 30 mM	75.85±4.44[a]	42.02±7.56[b]	33.19±5.88[a]
Glucose + Glycine	75.78±6.88[a]	42.20±5.83[b]	33.22±9.46[a]

Different superscripts in the same column indicate significant differences (P<0.05)

A drastic decrease in sperm motility occurred on the second day of storage in all treatment groups, ranging from 41.37-42.20%. However, the 30 mM glycine and glycine-glucose combination treatments showed statistically higher motility values (42.02% and 42.20%) compared to the control and glucose treatments (41.37% and 41.39%). This significant decrease in motility is a common phenomenon in the storage of liquid avian semen, caused by energy depletion, accumulation of metabolic products, and oxidative stress during storage (Santiago-Moreno et al., 2022). Glucose, as the primary energy source, is rapidly consumed by spermatozoa to maintain flagellar activity and cellular metabolic processes (Junaedi *et al*., 2024a). However, the addition of glycine appears to provide additional protective effects through plasma membrane stabilization mechanisms, thereby maintaining motility better than glucose alone.

On the third day of storage, the differences between treatments became more pronounced, with the 30 mM glycine (33.19%) and combination treatments (33.22%) maintaining significantly higher motility than the 30 mM glucose (31.26%). The glucose treatment showed the lowest motility on the third day, not significantly different from the control (32.13%). This finding indicates that glycine plays a crucial role in maintaining the viability of native chicken spermatozoa during short-term storage. Glycine reduces the formation of oxidants and increases sperm fertility because it is considered a synergistic antioxidant (Neamah, 2022). Furthermore, glycine also contributes to maintaining cell osmotic homeostasis, preventing excessive swelling or shrinkage that can compromise the integrity of the spermatozoa plasma membrane (Junaedi *et al*., 2023).

These results differ from previous studies that reported that the sperm yield of native chicken sperm was 78.33% after 24 hours of cold storage and decreased to 8.33% after 48 hours of cold storage using a diluent containing 20 mM glucose (Khaeruddin & Amir, 2019). Another study on native Sentul chicken sperm with a diluent containing 0.2% glucose showed better results, namely 67.08% after 24 hours of storage and 60.42% after 48 hours of storage (Khaeruddin *et al*., 2016).

The superiority of the glycine-glucose combination treatment in maintaining motility until the third day indicates a synergistic effect between the two components. Glucose provides an energy substrate for aerobic and anaerobic spermatozoa metabolism, while glycine provides structural and antioxidant protection. Glycolysis and oxidative phosphorylation of glucose produce adenosine triphosphate (ATP) as an energy source to maintain the motility of chicken spermatozoa (Setiawan *et al.*, 2020). This combination creates an optimal microenvironment for spermatozoa survival during refrigerated storage. This is thought to be related to glycine's ability to reduce hyperosmotic stress that can occur due to the addition of high glucose concentrations, while also facilitating more efficient glucose utilization by spermatozoa mitochondria.

The results of this study confirm that the addition of 30 mM glycine, either alone or in combination with glucose, effectively increases the motility of native chicken spermatozoa

during liquid semen storage for up to three days.. These findings have important practical implications for the development of artificial insemination technology for native chickens, particularly in the formulation of more effective and economical semen diluents. Further research is needed to evaluate other semen quality parameters such as viability, membrane integrity, and in vivo fertilization capacity, as well as to examine the molecular mechanisms behind glycine's protective effect on poultry spermatozoa.

Sperm Viability

The results showed that spermatozoa viability on the first day of storage was relatively uniform across all treatment groups, ranging from 87.03 to 88.32%, with no statistically significant differences. High initial viability values (>85%) indicate that semen collection, dilution, and handling were carried out appropriately, minimizing mechanical damage and initial osmotic stress. Consistent viability across all treatments also indicates that the addition of glucose, glycine, or a combination of both at a concentration of 30 mM did not cause any toxic or osmotic adverse effects on spermatozoa plasma membrane integrity. Higher viability than motility on the same day reflects that some spermatozoa still have intact plasma membranes despite progressive loss of motility, a common phenomenon in semen undergoing dilution and storage.

A significant decrease in spermatozoa viability occurred on the second day of storage, with values ranging from 65.29 to 67.92%. Treatment with 30 mM glycine (67.87%) and a combination of glycine and glucose (67.92%) maintained significantly higher viability compared to the control (65.38%) and 30 mM glucose (65.29%). This decrease in viability is closely related to plasma membrane damage due to oxidative stress (Kameni *et al*., 2021), exposure to low temperatures, can cause sub-lethal damage to spermatozoa, including agglutination of integral membrane proteins, changes in protein function, and loss of selective membrane permeability (Rizkallah *et al*., 2022). Glycine acts as a precursor of glutathione, a major endogenous antioxidant that protects spermatozoa membranes from reactive oxygen species (ROS) that accumulate during storage (Junaedi *et al*., 2024b).

On the third day of storage, the differences in viability between treatments became more pronounced, with the 30 mM glycine (57.89%) and combination treatments (57.90%) maintaining significantly higher viability compared to the control (54.53%) and 30 mM glucose (54.87%). The single glucose treatment showed no advantage over the control in maintaining sperm viability. This indicates that although glucose provides an energy substrate for sperm metabolism, without adequate membrane protection, viability continues to decline progressively. Conversely, glycine not only provides antioxidant protection but also plays a role in maintaining mitochondrial membrane potential and preventing premature sperm apoptosis through ion channel regulation and intracellular calcium homeostasis.

Table 2. Sperm viability of Kampung chickens using different liquid semen treatments during storage

Treatments	Viability (%)		
	Day 1	Day 2	Day 3
Control	87.05±6.53^{a}	65.38±6.37^{b}	54.53±4.52^{b}
Glucose 30 mM	88.32±4.67^{a}	65.29±4.54^{b}	54.87±7.76^{b}
Glycine 30 mM	87.03±5.86^{a}	67.87±8.89^{a}	57.89±8.45^{a}
Glucose + Glycine	87.08±3.44^{a}	67.92±6.46^{a}	57.90±5.88^{a}

Different superscripts in the same column indicate significant differences (P<0.05)

Comparison of viability and motility parameters in this study showed a consistent positive correlation, with treatments that maintained better viability also having higher motility. However, viability values were consistently 12–24% higher than motility values on each storage day, indicating the presence of a spermatozoa population with intact plasma membranes but loss of motility. Glycine and its combination treatments were able to slow this sequential degradation process by providing layered protection to various spermatozoa cellular components. The synergistic effect of the glycine-glucose combination creates an optimal microenvironment that provides adequate energy while protecting membrane structures from oxidative and osmotic damage.

The results of this study confirm that 30 mM glycine supplementation, either alone or in combination with glucose, effectively increases the viability of native chicken spermatozoa during liquid semen storage for up to three days. Maintained viability above 57% on the third day is still within the acceptable threshold for artificial insemination applications in poultry, although the fertilization success rate may be reduced compared to fresh semen. This result differs from previous findings that the viability of chicken sperm using a diluent containing glucose at 24 hours (second day) storage was 75.86% and 48 hours (third day) was 69.61% (Khaeruddin *et al*., 2016).

These findings provide scientific justification for the use of glycine as an essential component in native chicken semen diluent formulations, with potential for broader application in other poultry species. Further research is needed to assess other semen quality parameters such as acrosome integrity, DNA fragmentation, and capacitation status, as well as conducting in vivo fertility tests to validate the effectiveness of the treatment under field conditions.

CONCLUSION

Supplementation with 30 mM glycine and a 30 mM glycine-glucose combination significantly increased sperm motility and viability on the second and third days of storage compared to the control and single glucose treatments. The 30 mM glycine and 30 mM glycine-glucose combination treatments demonstrated superior protective properties in maintaining the quality of native chicken liquid semen for up to three days of storage, maintaining motility above 33% and viability above 57%.

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