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The Quality of Cryopreserved Semen of Local Chickens Treated with Ringer's Lactate-Egg Yolk Extender Supplemented with Glycine and Glucose

Junaedi[a,b], N. Isnaini[c], M. H. Natsir[c], & T. Susilawati[c,*]
[a]Doctoral Student in Animal Science, Faculty of Animal Science, University of Brawijaya
[b]Faculty of Agriculture, Fisheries, and Animal Husbandry, Universitas Sembilanbelas November Kolaka
[c]Department of Animal Science, Faculty of Animal Science, University of Brawijaya,
Jalan Veteran, Malang 65145, East Java, Indonesia
*Corresponding author: tsusilawati@ub.ac.id
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ABSTRACT

The aim of this study is to investigate the potential of glucose, glycine, and a combination of glucose and glycine supplemented into Ringer's lactate extender on the quality of pre- and post-cryopreservation of semen of local chickens. Semen samples were collected from KUB roosters. The research procedures involved formulation of the extender, semen collection, assessment of fresh semen, dilution, packaging, cryopreservation, storage, and subsequent assessment of cryopreserved semen. Parameters assessed in fresh, pre-cryopreservation, and post-thawed semen using computer-assisted semen analysis (CASA), included motility (total motility, progressive, static, and slow motilities), kinematics, spermatozoa morphology, malondialdehyde (MDA) concentration, and scanning electron microscopy (SEM). There were four treatments: T1= control, T2= 50 mM glucose, T3= 60 mM glycine, and T4= a combination of 50 mM glucose and 60 mM glycine. Each treatment was repeated 10 times. The results showed that the addition of 60 mM glycine and the combination of 50 mM glucose and 60 mM glycine effectively preserved total motility, maintained progressive motility, and reduced damage to the bent tail during the equilibration process (prior to cryopreservation). In addition, total motility, progressive motility, DAP, DSL, DCL, VAP, VSL, VCL, STR, and LIN were significantly increased after thawing. This approach also minimized the formation of MDA. Furthermore, observations using SEM showed comparatively more intact acrosomes compared to the other treatments. In conclusion, the addition of 60 mM glycine and a combination of 60 mM glycine and 50 mM glucose to Ringer's lactate-egg yolk extender proved to be effective in preserving the quality of domestic chicken spermatozoa during semen cryopreservation.

Keywords: CASA; cryopreserved semen; glucose; glycine; local chicken

INTRODUCTION

Indigenous chickens in Indonesia show great diversity. The local chicken breeds in Indonesia are diverse and include around 34 registered types (Henuk & Bakti, 2018). Effective breeding systems can be used to develop local chickens with higher genetic potential. The quality of inseminated semen is crucial to successful artificial insemination (AI) in chickens. Liquid semen offers a solution to the short shelf life of fresh semen at room temperature. However, liquid chicken semen can only be stored for about 3 days. Cryopreserved semen is a suitable alternative for long-term storage.

Cryopreservation at low temperatures (-196 °C) helps maintain both the structural and functional integrity of spermatozoa. Chicken semen cryopreservation can last up to 18 years in liquid nitrogen and has been shown to be highly effective in preserving genetic resources (Thélie *et al.*, 2019). Semen cryopreservation can be used in breeding programs to improve and enhance the efficiency of chicken farming (Woelders, 2021). Research on the cryopreservation of indigenous Indonesian chicken spermatozoa remains limited. Some of these studies are related to the Sentul chicken (Junaedi *et al.*, 2016), Kampung chickens (Khaeruddin *et al.*, 2020), and crossbred local chickens (Telnoni *et al.*, 2021). Cryopreserved chicken semen can be stored for a long period of time, but the quality of semen produced after thawing is often very low. In addition, cryopreservation of semen can affect membrane fluidity and acrosome reaction, which ultimately affects the fertilization ability of chicken spermatozoa (Nguyen *et al.*, 2015). Semen storage reduces spermatozoa metabolism to preserve their viability and fertility (Heydari *et al.*, 2021).

Ringer's lactate solution has been extensively researched in chicken semen. It consists of sodium, chloride, potassium, calcium, and lactate. This composition helps prevent cell death and supports cell survival (Fujita *et al.*, 2020). Ringer's lactate can be combined with egg yolk. Egg yolk contains low-

density lipoprotein (LDL), which is able to protect spermatozoa membranes from damage caused by storage (Manjunath, 2018). Glucose is an essential nutrient that can be incorporated into semen extenders. Glucose plays an important role as an energy source for spermatozoa. During this process, energy is generated in the form of ATP, which is used by spermatozoa for motility (Setiawan *et al.*, 2020).

Rooster spermatozoa are vulnerable to lipid peroxidation during cryopreservation (Nabi *et al.*, 2016). Free radicals are highly reactive and can react with these unsaturated fatty acids, making them susceptible to damage caused by lipid peroxidation. Antioxidants in semen play a crucial role in preventing damage due to oxidative stress. However, the levels of antioxidants in semen are reported to decrease during dilution and storage (Kutluyer & Kocabas, 2016). Amino acids can act as antioxidants. Glycine is one of the amino acids that can be added to semen cryopreservation extenders. The use of glucose, glycine, and the combination of glucose and glycine supplemented into the Ringer's lactate base extender to maintain the quality of cryopreserved semen in Indonesian domestic chickens has not been previously explored. Therefore, this study is conducted to evaluate the effect of glycine and glucose on the quality of pre- and post-cryopreservation semen of local chickens.

MATERIALS AND METHODS

Research Materials

The semen materials used in this study were obtained from local KUB roosters. KUB chickens are a local breed developed at the Livestock Research Center BALITNAK to be utilized for egg-laying and meat production. The Agricultural Research and Development Agency is an Indonesian governmental institution responsible for the production of high-quality agricultural products. Research has been conducted through BALITNAK, leading to the development of superior local livestock products, such as KUB chicken, which is known for its high egg and meat production capabilities. The roosters used were 12 months old. The chickens used are in puberty and reach maturity. The feed provided is a complete pellet produced by PT. Indonesian Gold Coin. Feed is given at a rate of 200 g/day, and drinking water is given *ad libitum*. The nutritional contents of the feed are presented in Table 1.

Table 1. Nutrient content of feed

Composition	Nutrient (%)
Crude protein	17.00
Ash	14.00
Crude fiber	6.00
Crude fat	3.00
Calcium	4.20
Phosphorus	0.60

Note: Source from the company PT. Indonesian Gold Coin.

The procedures, mechanisms, and entire series of research activities have been evaluated and approved by the Animal Care and Use Committee, Brawijaya University, Malang, East Java, Indonesia, 19 June 2023, with certificate number 056-KEP-UB-2023. Semen collection is carried out in the morning starting at 07.30 using massage techniques. Semen is obtained by placing the left hand on the back of the chicken and massaging around the cloaca with two fingers until the papilla protrudes. Protrusion of these papillae is followed by ejaculation of fresh semen.

Research Procedures

Preparation of liquid semen extender. The basic extender used was ringer lactate (PT. Widatra Bakti, Indonesia) containing 1.55 g of sodium lactate, 3 g of sodium chloride, 0.15 g of potassium chloride, and 0.1 g of calcium chloride in 500 mL sterile water, with osmolarity of 274 mOsm L -1). The base extender material used consisted of 90% Ringer's lactate solution and 10% egg yolk. It was then centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was utilized as the foundational extender. This supernatant was collected and enriched with penicillin (1000 IU/mL) (PT. Meiji, Indonesia), streptomycin (1 mg/mL) (PT. Meiji, Indonesia), and DMSO (7%) (Merck, KgaA, Darmstadt Germany). The extender's pH was adjusted to 7.4 using Tris (hydroxymethyl aminomethane) (Merck, KgaA, Darmstadt Germany). The extender was prepared according to specific treatments (T1= control, T2= 50 mM glucose (Merck, KgaA, Darmstadt Germany), T3= 60 mM glycine (Merck, KgaA, Darmstadt Germany), and T4= a combination of 50 mM glucose and 60 mM glycine). Subsequently, each treatment was distributed into four tubes and homogenized. The extender's composition can be observed in Table 2.

Collection and evaluation of fresh semen. Semen collection was conducted through the massage method three times a week. Fresh semen underwent analysis utilizing an advanced electronic imaging system for visualizing spermatozoa via computer-assisted semen analysis (CASA). Testing of chicken spermatozoa quality using CASA was carried out at the Artificial Insemination Seed Center (BBIB) in Singosari, Malang, East Java, Indonesia. Variables observed included motility (i.e., total, progressive, statics, and slow motilities), sperm kinematics (i.e., the distance of the curved line [DCL], the distance of the straight line [DSL], the distance of the average path [DAP], the velocity of the curved line [VCL], the velocity of the average pathway [VAP], the velocity of the straight line [VSL], linearity [LIN], straightness [STR], wobble [WOB], beat cross frequency [BCF], amplitude of lateral head [ALH], and average orientation and change of head [AOC]), and spermatozoa morphology (i.e., bent tail, coiled tail, DMR, distal droplet, and proximal droplet).

Dilution, packaging, cryopreservation, storage, and evaluation of cryopreserved semen. The collected

Table 2. Composition of treatment diluent materials

Diluent composition	Treatment			
	Control	Glucose	Glycine	Combination (Glucose + Glycine)
RLKT (mL)	9.3	9.3	9.3	9.3
DMSO (mL)	0.7	0.7	0.7	0.7
Glucose (mM)	0	50	0	50
Glycine (mM)	0	0	60	60
Penicillin 1000 IU/mL	0.01	0.01	0.01	0.01
Streptomycin 1 mg/mL	0.01	0.01	0.01	0.01
Total (mL)	10	10	10	10

Note: RLKT (Ringer's Lactate Supernatant 90% and Egg Yolk 10%), 50 mM= 0.09 grams of glucose, 60 mM= 0.045 grams of glycine, pH of the diluent is adjusted/adjusted (7.4) with Tris hydroxymethyl aminomethane

semen was diluted using treatment extenders (control, 50 mM glucose, 60 mM glycine, and combination [50 mM glucose + 60 mM glycine]) at a 1:5 ratio. Subsequently, 0.25 mL straws (IMV, France) were filled with the liquid semen, followed by equilibration at 5 °C for 2 hours. The liquid semen was evaluated before the initial cryopreservation stage. The first cryopreservation stage involved placing straws containing semen 3 cm above the liquid nitrogen surface (Madeddu *et al.,* 2016), for 10 minutes (Mosca *et al.,* 2016). Afterward, the straws were immersed in liquid nitrogen for the storage of cryopreserved semen (second cryopreservation stage and storage process). The cryopreserved semen was stored in a liquid nitrogen container (-196 °C) for subsequent testing. The evaluation of cryopreserved semen was conducted by thawing it in warm water (37 °C) for 30 seconds.

Research Parameters

The observed parameters during fresh semen, pre-Cryopreservation, and post-thawing stages were as follows:

Computer-Assisted Semen Analysis (CASA) parameters. Motility parameters included (1) total, (2) progressive, (3) static, and (4) slow motilities. Meanwhile, kinematic parameters consisted of the following: (1) VAP (the velocity of the average path, μm/second) represents the average speed of spermatozoa along their pathway; (2) VSL (the velocity of the straight line, μm/second) indicates the average speed of spermatozoa in a straight line from start to finish; (3) VCL (the velocity of the curved line, μm/second) reflects the average speed at each movement point along the pathway; (4) DCL (the distance of the curved line, μm) signifies the distance traveled by spermatozoa in one minute on a curved path; (5) DSL (the distance of the straight line, μm) denotes the distance covered by spermatozoa in one minute along a straight path; (6) DAP (the distance of the average path, μm) describes the distance traveled by spermatozoa in one minute on an average path; (7) ALH (amplitude of lateral head, μm) measures the lateral movement distance of spermatozoa along their average path; 8) LIN (linearity, %) indicates the degree of linearity on the curved path (VSL divided by VAP); (9) STR (straightness, %) represents the linearity of the average path (derived from VSL divided by VAP); (10) BCF (beat cross frequency, hertz) calculates the average frequency of spermatozoa crossing their average path; (11) WOB (wobble, %) measures the actual trajectory oscillation, indicating the strength of spermatozoa movement in one second. Apart from that, the spermatozoa morphology parameters included (1) bent tail, (2) coiled tail, (3) DMR, (4) distal droplet, and (5) proximal droplet.

Analysis of Malondialdehyde (MDA) concentration after cryopreservation (post-thawing). Cryopreserved chicken semen was initially thawed in a water bath (37 °C) for 30 seconds. The semen was then extracted from the straw, dropped into an Eppendorf tube, and 250 μm of semen was mixed with 625 μm of 40% TCA (Kheawkanha et al., 2023), 100 μm of 1N HCl, 50 μm of 1% Na Thio, and 975 μm of ddH_2O, so that the volume of semen was 2000 μm (2 mL). The sample was then heated at 100 °C for 30 minutes. It was then centrifuged at 4000 rpm for 10 minutes (Mossa *et al.,* 2012). The resulting supernatant was collected and supplemented with ddH_2O to achieve a total sample volume of 3 mL. Spectrophotometric measurements were performed at a wavelength of 532 nM (Kheawkanha *et al.,* 2023). The MDA concentration was then measured and expressed in nmol/mL.

Testing semen quality after cryopreservation using a Scanning Electron Microscope (SEM). Thawed semen samples were mixed with 1 mL of saline buffer phosphate and then fixed using glutaraldehyde for 1 hour. Following this, the samples were washed with phosphate buffer at pH 7.4 three times for 5 minutes each. The samples were placed on a covered glass and subjected to a gradual dehydration process: immersed successively in 50% alcohol for 5 minutes, 70% alcohol for 5 minutes, 80% alcohol for 5 minutes, 90% alcohol for 5 minutes, and finally in absolute alcohol for 5 minutes. The cover glass loaded with the sample was then coated with platinum using the Q 15RS coating equipment. The prepared samples were examined using a Hitachi TM 3000 scanning electron microscope (Akhtar, 2018).

Data Analysis

Statistical analysis was conducted by using SPSS 21.0. The research process was carried out using a completely randomized design (CRD) with 10

replications. The mathematical model is as follows:

$$Yij = \mu + \alpha i + \varepsilon ij$$

Where Yij is the response obtained from the influence of the i-th treatment and j-th replication, μ is the general average value, αi is the effect of treatment i, and εij is the effect of error on the ith treatment and the jth replication.

RESULTS

Characteristics of Fresh Semen from Local Chickens

The qualities of fresh semen of local chickens observations using CASA (computer assisted semen analysis) are presented in Table 3. The motilities of fresh semen from domestic chickens comprise a total motility of 88.38±5.56%, progressive motility of 64.16±7.04%, static motility of 11.62±5.61%, and slow motility of 6.18±2.49%. The parameter that determines the success of spermatozoa in fertilizing an egg cell is progressive motility. Spermatozoa are considered immobile if they move at an average path velocity of less than 5 um/s. On the other hand, they are classified as exhibiting progressive movement when their average path velocity exceeds 20 um/s with a linearity index of 80 (Kheawkanha *et al.,* 2023). Progressive motility refers to spermatozoa swimming swiftly in a straight line. It serves as an indicator of actively viable spermatozoa. The tail of the spermatozoa plays a crucial role in facilitating progressive motility. It contains an axoneme covered by the plasma membrane and is responsible for driving the progressive movement of spermatozoa. Damage to the tail part of spermatozoa leads in the loss of their motility.

Table 3. Characteristics of fresh semen from local chickens using computer assisted semen analysis (CASA)

Variables		Mean±sd
Motility	Total motile (%)	88.38±5.56
	Progressive (%)	64.16±7.04
	Statics (%)	11.62±5.61
	Slow (%)	6.18±2.49
Kinematics	DAP (μm)	32.81±4.56
	DSL (μm)	24.92±3.78
	DCL (μm)	49.26±6.45
	VAP (μm/s)	94.16±9.78
	VSL (μm/s)	74.51±9.82
	VCL (μm/s)	139.67±15.93
	STR (%)	94.16±9.78
	LIN (%)	51.90±10.07
	ALH (μm)	7.10±1.29
	BCF (Hz)	27.58±3.07
	WOB (%)	66.18±6.57
Morphology	Bent tail	0.88±1.98
	Coiled tail	0.03±0.07
	DMR	0.40±0.62
	Distal droplet	0.12±0.21
	Proximal droplet	0.00±0.00

Note: DAP= Distance average path; DSL= Distance straight-line; DCL= Distance curve-line; VAP= Velocity average pathway; VSL= Velocity straight line; VCL= Velocity curve linear; STR= Straightness; LIN= Linearity; ALH= Amplitude lateral head; BCF= Beat cross frequency; WOB= Wobble.

Characteristics of Local Chicken Semen Before Cryopreservation

The qualities of local chicken spermatozoa before cryopreservation (after 2 hours of equilibration), using the basic extender Ringer's lactate egg yolk with the addition of glucose, glycine, and the combination (glucose + glycine) are presented in Table 4.

The research findings on total, progressive, static, and slow motilities before cryopreservation of local chicken semen showed significant differences ($p<0.05$) among the treatments. The addition of 60 mM glycine and the combination of 50 mM glucose and 60 mM glycine were able to maintain total and progressive motilities. Static motility was significantly lower ($p<0.05$) in extenders containing glycine and the combination of glucose and glycine. Supplementation of 60 mM glycine and the combination of 60 mM glycine and 50 mM glucose still maintained total motility (>80%) and progressive motility of liquid semen (>40%) after a 2-hour equilibration period. Equilibration is part of the semen cryopreservation process, involving storing liquid semen for 2 hours at a cool temperature (5 °C) before cryopreservation.

The kinematic assessment of spermatozoa before cryopreservation showed no difference among the treatments. However, there were variations ($p<0.05$) in the semen morphology before cryopreservation, particularly in bent tail damage, while no differences were observed among the treatments regarding coiled tail, DMR, distal droplet, and proximal droplet. The combination of glucose and glycine in Ringer's lactate base extender effectively reduced bent tail damage (0.19±0.15%). Conversely, treatments using only glucose or glycine individually resulted in higher bent tail damage.

Characteristics of Local Chicken Semen After Cryopreservation (Post-Thawing)

The qualities of local chicken spermatozoa after cryopreservation are shown in Table 5. The total motilities of local chicken spermatozoa after cryopreservation showed no differences among the treatments. However, the treatments differed ($p<0.05$) in progressive, static, and slow motilities. The addition of 60 mM glycine and the combination of 50 mM glucose and 60 mM glycine in the extender produced sperms that performed significantly better ($p<0.05$) than the addition of glucose alone and the control. Extenders supplemented with glycine and the combination (glycine + glucose) maintained progressive motility and minimized static and slow motilities. The total motilities (70.25±10.97%; 68.52±8.27%) with the addition of 60 mM glycine and the combination (50 mM glucose + 60 mM glycine) were higher than the use of hyaluronic acid, which reported a total motility of 55.3±1.1% in Lotfi *et al*. (2017), higher than the reports of Partyka *et*

Table 4. Quality of local chicken spermatozoa before cryopreservation (after 2 hours equilibration)

Variables		Treatments			
		Control	Glucose	Glycine	Glycine + Glucose
Motility	Total motile (%)	75.50±7.60[a]	80.3410.69[ab]	85.46±8.85[bc]	89.72±5.42[c]
	Progressive (%)	38.42±12.75[a]	39.29±16.94[a]	42.02±9.24[ab]	53.23±11.20[b]
	Statics (%)	24.50±7.60[a]	19.66±10.69[ab]	14.54±8.85[bc]	10.28±5.42[c]
	Slow (%)	19.40±9.93	17.71±5.42	16.97±8.25	14.74±7.22
Kinematics	DAP (μm)	25.21±9.10	25.37±12.84	23.62±7.31	27.41±5.74
	DSL (μm)	22.21±9.61	22.32±12.94	20.57±6.80	24.50±5.80
	DCL (μm)	36.97±8.57	36.74±12.74	34.79±7.85	38.79±5.83
	VAP (μm/s)	61.29±19.68	64.29±28.35	60.82±18.05	74.96±21.30
	VSL (μm/s)	55.63±19.29	56.71±28.70	55.32±24.25	66.69±21.63
	VCL (μm/s)	91.22±17.78	93.37±27.76	91.59±20.75	104.63±21.98
	STR (%)	62.05±18.72	64.29±28.35	61.82±17.81	74.96±21.30
	LIN (%)	51.74±20.19	52.97±11.50	54.84±5.43	58.49±5.92
	ALH (μm)	4.43±0.53	4.48±0.45	4.75±0.40	4.81±0.61
	BCF (Hz)	29.50±3.18	29.58±3.18	28.06±2.80	29.13±2.89
	WOB (%)	62.27±10.29	62.44±9.31	63.61±4.24	66.52±4.59
Morphology	Bent tail	0.24±0.16[a]	0.63±0.45[b]	0.33±0.50[ab]	0.19±0.15[a]
	Coiled tail	0.08±0.18	0.04±0.08	0.04±0.10	0.02±0.06
	DMR	4.01±2.15	3.69±3.12	4.36±2.95	1.95±1.65
	Distal droplet	0.16±0.25	0.15±0.22	0.10±0.12	0.02±0.04
	Proximal droplet	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). DAP= Distance average path; DSL= Distance straight-line; DCL= Distance curve-line; VAP= Velocity average pathway; VSL= Velocity straight line; VCL= Velocity curve linear; STR= Straightness; LIN= Linearity; ALH= Amplitude lateral head; BCF= Beat cross frequency; WOB= Wobble.

Table 5. Quality of local chicken spermatozoa after cryopreservation

Variables		Treatments			
		Control	Glucose	Glycine	Glycine + Glucose
Motility	Total motile (%)	55.79±22.13	59.21±59.21	70.25±10.97	68.52±8.27
	Progressive (%)	3.96±2.03[a]	11.87±17.93[a]	28.03±15.14[b]	34.45±12.34[b]
	Statics (%)	46.21±20.59[a]	40.78±22.18[ab]	29.75±10.97[b]	31.47±8.27[ab]
	Slow (%)	30.70±14.66[a]	26.95±14.23[ab]	21.23±8.70[ab]	18.28±4.60[c]
Kinematics	DAP (μm)	9.04±1.83[a]	14.71±11.00[a]	27.60±15.92[b]	31.01±13.93[b]
	DSL (μm)	6.47±1.73[a]	12.27±10.72[a]	25.03±16.05[b]	27.77±14.44[b]
	DCL (μm)	19.43±4.68[a]	24.23±12.16[a]	37.19±15.52[b]	41.71±12.77[b]
	VAP (μm/s)	22.63±4.98[a]	35.78±26.80[a]	65.73±35.55[b]	79.26±31.96[b]
	VSL (μm/s)	16.36±3.78[a]	29.88±26.18[a]	58.39±35.68[b]	69.26±37.09[b]
	VCL (μm/s)	47.81±12.81[a]	58.39±29.83[a]	90.78±34.79[b]	108.96±28.19[b]
	STR (%)	22.63±4.98[a]	35.78±26.80[a]	66.83±35.37[b]	80.26±31.35[b]
	LIN (%)	38.87±11.46[a]	44.95±11.51[ab]	55.68±12.40[b]	56.06±11.96[b]
	ALH (μm)	3.26±0.88	2.80±80.54	3.97±0.87	5.03±0.60
	BCF (Hz)	35.76±4.24[a]	34.91±3.37[a]	31.49±3.04[b]	30.43±3.39[b]
	WOB (%)	49.45±8.17	54.71±9.22	59.46±21.55	65.25±9.11
Morphology	Bent tail	0.41±0.51	0.51±0.57	0.74±0.70	0.70±0.87
	Coiled tail	0.04±0.05	0.03±0.07	0.00±0.00	0.05±0.13
	DMR	6.88±2.74	5.08±3.47	5.02±3.71	4.93±2.60
	Distal droplet	0.14±0.19	0.14±0.19	0.18±0.28	0.28±0.37
	Proximal droplet	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). DAP= Distance average path; DSL= Distance straight-line; DCL= Distance curve-line; VAP= Velocity average pathway; VSL= Velocity straight line; VCL= Velocity curve linear; STR= Straightness; LIN= Linearity; ALH= Amplitude lateral head; BCF= Beat cross frequency; WOB= Wobble.

al. (2017) (49.06%) for indigenous chicken spermatozoa using an EK extender with 1 mM L-carnitine, and higher than Fattah *et al*. (2017) report of 68.2% with the addition of 1 mM L-carnitine in the Beltsville extender. The progressive motility observed upon the addition of glycine (28.03±15.14%) and the combination of glycine with glucose (34.45±12.34%) was higher compared to the progressive motility reported in several studies: 25.2

± 0.8% in Lotfi *et al*. (2017), 28.4% in Fattah *et al*. (2017), 16.48% in Partyka *et al*. (2017), and 24% using Sasaki extender supplemented with 1 mM L-carnitine as noted in Kumar *et al.* (2019). The motility percentage decreases after cryopreservation of chicken semen, as chicken spermatozoa are easily damaged (Junaedi *et al.*, 2017).

Analysis of the quality of chicken semen after cryopreservation in relation to the kinematic parameters revealed no differences among treatments for ALH and WOB parameters. However, there were significant differences ($p<0.05$) in DAP, DSL, DCL, VAP, VSL, VCL, STR, LIN, and BCF. Treatment with 60 mM glycine and the combination of 50 mM glucose and 60 mM glycine increased DAP, DSL, DCL, VAP, VSL, VCL, STR, and LIN. In addition, the treatments with 60 mM glycine and the combination had significantly lower BCF values ($p<0.05$) compared to the control and the addition of 50 mM glucose. The addition of 60 mM glycine and the combination (glucose 50 mM + glycine 60 mM) to Ringer's lactate-egg yolk base extender boosted VAP and VCL. The VCL measures the average velocity in the actual point-to-point pathway followed by spermatozoa. The VCL obtained was 90-108 μm/s, with VAP at 65-79 μm/s. Compared to the standard quail spermatozoa velocity of 45 μm/s (Farooq *et al.*, 2017), the velocities in this study were relatively high. Supported by Sayed *et al*. (2017), chicken spermatozoa are considered fast if VAP is above 50 μm/s. Morphologically, there were no differences after cryopreservation in terms of bent tail, coiled tail, DMR, distal droplet, and proximal droplet.

Malondialdehyde (MDA) Concentration

Malondialdehyde (MDA) is an aldehyde compound that results from lipid peroxidation and is toxic to spermatozoa. The concentrations of MDA in local chicken spermatozoa after cryopreservation (after-thawing) using Ringer's lactate-egg yolk base extender supplemented with glucose, glycine, and a combination of both (glucose + glycine) are presented in Figure 1.

The results showed that the combination treatment (glycine + glucose) added to Ringer's lactate-egg yolk base extender performed better ($p<0.05$) than the control and the addition of glucose alone. The combination treatment (60 mM glycine + 50 mM glucose) effectively minimized MDA formation to 0.85±0.17 nmol/mL. The concentration of MDA serves as a measure, indicating lipid peroxidation that occurs in semen during storage (Kheawkanha *et al.*, 2023). MDA compounds cause damage to spermatozoa membranes and a decrease in membrane integrity, which impairs sperm quality. Storing of semen in a cryopreserved state affects the MDA level. Glycine, an amino acid contained in glutathione, reduces oxidative stress and enhances the activity of antioxidant enzymes (Zhou *et al.*, 2018).

Observation of Local Chicken Spermatozoa After Cryopreservation Using a Scanning Electron Microscope (SEM)

The observation of local chicken spermatozoa after cryopreservation using a SEM revealed that the combination of 60 mM glycine and 50 mM glucose in Ringer's lactate-egg yolk extender showed more intact acrosomes compared to the control (Figure 2). The acrosomal cap is a sheath on the head of the spermatozoa that functions to protect the release of genetic material and enzymes. The acrosomal cap plays a crucial role in fertilization success during mating. Its role in the fertilization process is related to the level of enzymes it contains. Damage to the acrosomal cap can lead to the release of enzymes, resulting in the loss of the spermatozoa's ability to fertilize. The integrity of the spermatozoa's acrosomal cap is an important parameter that indicates successful fertilization, as it contains acrosomal enzymes responsible for lysing cumulus cells when the spermatozoa meet the egg.

DISCUSSION

Spermatozoa motility is a decisive factor for fertility in chickens. It correlates closely with the viability of spermatozoa. A low percentage of spermatozoa motility leads to the reduced viability. Conversely, a higher percentage of spermatozoa motility leads to a higher percentage of viability. Higher spermatozoa motility per-

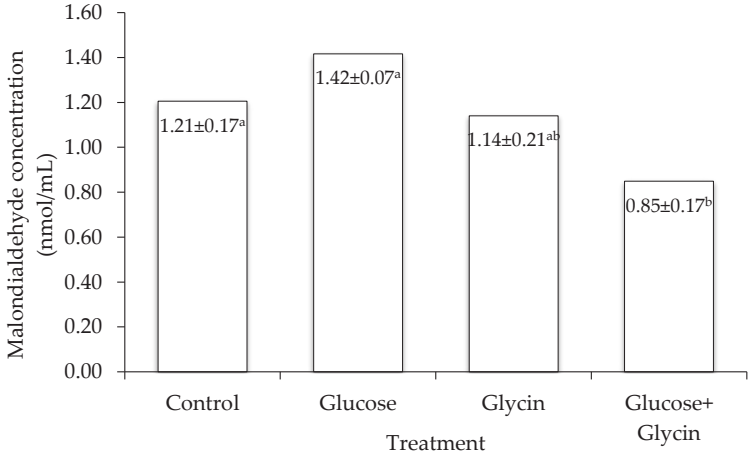

Figure 1. Malondialdehyde concentration (nmol/mL) of local chicken spermatozoa after cryopreservation (post-thawing). Different superscripts differ significantly at $p<0.05$.

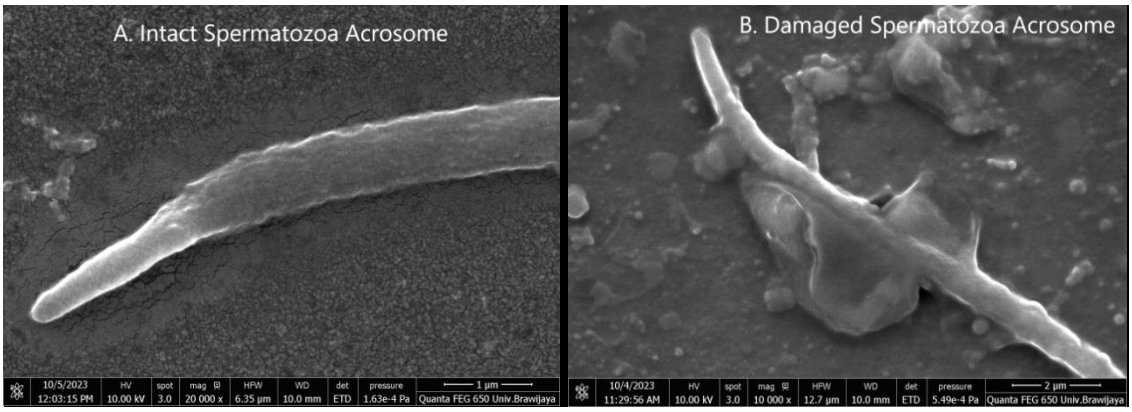

Figure 1. Acrosome cap and spermatozoa membrane local chicken after cryopreservation, A) Acrosome cap and spermatozoa membrane that are intact after cryopreservation using a combination of diluent (glycine 60 mM + glucose 50 mM), and B) The acrosome cap and spermatozoa membrane after cryopreservation are damaged (without the addition of glycine and glucose in the semen diluent).

centages suggest progressive and cohesive movement, indicating a larger population of viable spermatozoa. Semen with a lower percentage of spermatozoa motility have poorer survivability. Spermatozoa motility has been reported to be an important factor in the successful journey of spermatozoa to the sperm storage tubules (SST) (Kheawkanha *et al.,* 2021). Spermatozoa motility significantly impacts male fertility as spermatozoa must traverse the female reproductive tract to reach and fertilize the oocyte. Motility is crucial for reaching the oocyte (Moreno & Lucero, 2019). Motility assessment is a primary and straightforward evaluation of artificial insemination.

Equilibration involves storing liquid spermatozoa at a cold temperature (5 ^{o}C) for 2 hours before cryopreservation. The equilibration is intended to prevent the spermatozoa from coming under cold stress during the semen cryopreservation process. Keeping spermatozoa in a liquid state at low temperatures (2-5 °C) is necessary to slow down their metabolism, allowing them to survive longer (Fattah *et al.,* 2017). Nevertheless, as Blank *et al*. (2021) report, spermatozoa motility decreases in liquid semen, indicating a significant decrease in motility after storage. Storage leads to changes in semen quality due to the increased oxidative stress, which can reduce motility (Víquez *et al.,* 2020). The reduction in motility of liquid semen is also caused by the ongoing cellular movement and metabolism during storage.

This study shows that adding glycine and the combination of glucose and glycine to Ringer's lactate-egg yolk extender improves total and progressive motility while reducing the percentage of slow and static motilities. Glycine, an amino acid that acts as an antioxidant, helps maintain the quality of spermatozoa during semen storage. Antioxidants protect spermatozoa from free radical attacks that cause lipid peroxidation. These results are consistent with the studies of Khaeruddin *et al*. (2022) and Iswati *et al*. (2018), which showed that supplementing chicken semen with antioxidants maintains spermatozoa quality. Lipid peroxidation is one of the reasons for the reduced motility of chicken spermatozoa (Mussa *et al.,* 2021). The addition of glucose to the glycine combination positively affects the maintenance of spermatozoa quality after cryopreservation. Glucose provides an additional source of energy for the spermatozoa during storage. In addition, the progressive motility of chicken spermatozoa depends to a considerable extent on the total energy production originating from the mitochondrial compartment. Chicken spermatozoa motility positively correlates with mitochondrial respiratory enzyme activity and adenosine triphosphate (ATP) (Sangani *et al.,* 2017). ATP is generated by glycolysis and the oxidative phosphorylation of glucose, serves as an energy source to maintain motility of chicken spermatozoa (Setiawan *et al.,* 2020). Stanishevskaya *et al*. (2021) reported that simple sugar-derived carbohydrates easily penetrate the chicken spermatozoa membrane and create additional energy reserves to ensure spermatozoa motility. Kuzlu & Taşkin (2017) suggested that glucose-based extender is a suitable option for the long-term storage of turkey sperm.

High MDA concentrations in spermatozoa and seminal plasma after cryopreservation result from ROS that attack lipids in the spermatozoa membrane. Chicken spermatozoa membranes contain high concentrations of polyunsaturated fatty acids (PUFA) (Mussa *et al.,* 2021), which makes them susceptible to lipid peroxidation (LPO) due to the presence of ROS during storage. Seminal plasma, the primary source of antioxidants for defense mechanisms, was exposed to aerobic conditions during the pre-cryopreservation process. As a result, spermatozoa have fewer antioxidants to protect them from unfavorable ROS activities, leading to the increased oxidative stress that initiates lipid peroxidation and results in the formation of MDA.

The cryopreservation of semen causes ultrastructural, biochemical, and functional damage to spermatozoa. Various spermatozoa organelles are susceptible to cryopreservation effects, resulting in altered mitochondrial function, failure of chromatin decondensation, and premature triggering of the acrosomal reaction due to changes in plasma membrane permeability. These changes can impair spermatozoa viability and fertility. The damage is caused by forming

extracellular ice crystals from the water in the semen-cryopreservation medium. This formation increases the concentration of dissolved substances, such as sugars, salts, and proteins. Reactive oxygen species during storage lead to DNA damage in spermatozoa, reduced mitochondrial function, and decreased adenosine triphosphate (ATP) production (Sangani *et al.,* 2017; Masoudi *et al.,* 2019; Cheng *et al.,* 2015). The addition of an antioxidant in semen supplements is essential for maintaining optimal spermatozoa quality during semen cryopreservation.

CONCLUSION

The addition of 60 mM glycine and the combination of 50 mM glucose and 60 mM glycine significantly increased total motility, progressive motility, DAP, DSL, DCL, VAP, VSL, VCL, STR, LIN, and minimized the formation of MDA after-thawing. Observations using SEM revealed comparatively more intact acrosomes compared to the other treatments. The addition of 60 mM glycine and a combination of 60 mM glycine and 50 mM glucose to Ringer's lactate-egg yolk extender proves effective in maintaining the quality of local chicken spermatozoa during semen cryopreservation.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with any other people or organization related to the material covered in the manuscript.

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