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The Quality of Gaga Roosters Semen During Cold Storage Using a Diluent Supplemented with Sorbitol

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ABSTRACT

This study aimed to examine the effect of adding different concentrations of sorbitol to the Ringer's lactate-egg yolk (RLEY) diluent on the quality of Gaga roosters' semen during cold storage. A completely randomized design was used with 10 replicates and 4 levels of diluent treatment, including control, 1%, 2%, and 3% sorbitol. Semen was collected using a massage method, followed by fresh semen evaluation and dilution with a ratio of 1:5. Semen was stored at a cold temperature (5 ºC) and observed after 0, 24, 48, and 72 hours. The variables observed were semen pH, motility, kinematics, viability, plasma membrane integrity, acrosome integrity, malondialdehyde (MDA), and intracellular calcium ion (Ca^{2+}) concentrations of sperm. All data were analyzed using analysis of variance (ANOVA). Subsequently, the evaluation of sperm mitochondrial activity and DNA damage was carried out, and the data were analyzed using the T-test. The results showed that treatment with sorbitol in the diluent had no effect on semen pH, kinematics (except straightness), acrosomal cap integrity, DNA damage, mitochondrial activity, and MDA concentration. However, sorbitol concentrations significantly affect ($p<0.05$) total motility, progressive motility, static motility, STR, viability, plasma membrane integrity, and intracellular Ca^{2+}. In conclusion, the addition of 1-3% sorbitol preserves the quality of Gaga roosters' semen during 72 hours of cold storage.

Keywords: cold storage; diluent; Gaga rooster; semen; sorbitol

INTRODUCTION

Keeping local ornamental chickens as a hobby provides pleasure and entertainment for poultry lovers and contributes to the preservation and development of unique attractive chickens' breeds, some of which have high genetic and historical value. The Gaga is a local Indonesian chicken breed from South Sulawesi known for its long laugh-like sound, earning it the nickname "laughing chickens" (Bugiwati & Ashari, 2013). Breeders keep these breeds as ornamental chickens that often participate in competitions. Gaga roosters that have won competitions fetch a high selling price, thus potentially generating income for traditional breeders (Khaeruddin *et al.*, 2024b). By raising awareness of the importance of conserving Gaga roosters and supporting conservation efforts, all stakeholders can ensure the preservation of valuable cultural heritage and the sustainability of traditional farming communities.

The application of artificial insemination (AI) technology in poultry production has facilitated the expeditious dissemination of genetic material from a restricted cohort of superior males to a vast population of females (Mohan *et al.*, 2018). The use of AI allows for the semen from a single rooster to be used to inseminate up to ten times as many females as would be achieved through natural mating (Taye & Esatu, 2022). The technology enhances the productivity of chickens by facilitating the utilization of genetically high-quality superior roosters (Kheawkanha *et al.*, 2023). The aforementioned wider utilization of semen can be achieved through the implementation of cold storage. Balogun (2021) posts that liquid storage is more reliable for AI than frozen storage. The objective of storing

semen at a temperature between 4 °C and 10 °C is to decelerate the metabolic rate of sperm, thus extending their longevity (Gibb & Aitken, 2016).

The storage of semen has been demonstrated to result in the impairment of sperm, both metabolically and structurally (Partyka & Niżański, 2022). To ensure proper function, an energy source must be added to the diluent (Getachew *et al.*, 2015). Therefore, sugar and sugar alcohols (polyols) play an important role as energy reserves and also help maintain the structural integrity of sperm (Silyukova *et al.*, 2022). In this context, fructose, a sugar, serves as an energy source and can improve *in vitro* motility and longevity in roosters' sperm (Getachew *et al.*, 2015).

Sorbitol, a polyol, can be converted into fructose, which serves as an important energy source (Cao *et al.*, 2009). As Wu *et al.* (2016) stated, polyol is more effective than fructose and glucose in maintaining the quality of sheep sperm. In addition to serving as an energy source (Conrozier *et al.*, 2018), sorbitol has been demonstrated to function as an antioxidant (Soostuwan *et al.*, 2013), a membrane stabilizer, and a media osmolality regulator (Chandler-Brown *et al.*, 2015). Previous reviews have demonstrated that the addition of 2% sorbitol to Ringer lactate egg yolk diluent can improve motility, viability, plasma membrane integrity, and mitochondrial activity while also reducing the levels of lipid peroxidation in frozen semen from Gaga roosters (Khaeruddin *et al.*, 2024b). Consequently, this study introduces several pioneering elements, including the inaugural investigation of the impact of sorbitol on sperm quality and intracellular Ca^{2+} concentrations during cold storage, as well as the first to characterize the kinematics of local Indonesian roosters' sperm during cold storage. The objective of the analysis is to ascertain the impact of incorporating varying concentrations of sorbitol into the diluent on the quality of Gaga roosters' semen during cold storage.

MATERIALS AND METHODS

Ethical Approval

The implementation of this study has been permitted by the Brawijaya University Research Ethics Commission with number 020-KEP-UB-2023.

Research Procedures

Animals' maintenance and semen collection. The subjects, ages approximately 10 months were housed in individual cages measuring 55 x 60 x 60 cm^2. They were provided with a complete feed containing 17% crude protein, 3% crude fat, 7% crude fiber, and 14% ash, as much as 150 g/head/day, and water. Drinks are provided in unlimited quantities. Semen was collected from 5 roosters using the massage method and subsequently transferred to the laboratory for proper dilution. According to Kucera & Heidinger (2018), the method entailed massaging the cloaca until an erection was achieved, which was then followed by ejaculation. Subsequently, semen was collected using a tuberculin syringe.

Diluent preparation. The basic diluent employed was a supernatant comprising 90% Ringer's lactate and 10% chicken egg yolk. The composition of Ringer lactate (Widatra Bakti, Indonesia) consisted of 3 g NaCl, 1.55 g $NaC_3H_5O_3$, 0.15 g KCl, and 0.1 g CaCl in 500 mL of sterile water. The suspension was subjected to centrifugation at 1008 rcf for a period of 15 minutes. The supernatant was then collected and combined with 1000 IU/mL Penicillin (PT. Meiji, Indonesia) and 1 mg/mL Streptomycin (PT. Meiji, Indonesia). The pH level of the solution was adjusted to attain a value of 7.8 through the addition of tris hydroxyl aminomethane (Merck KGaA, Germany). Subsequently, the diluent was divided into four tubes containing a distinct concentration of sorbitol (Merck KGaA, Germany), as indicated in Table 1.

Dilution, storage, and evaluation of semen. The fresh semen was evaluated, and sperm motility above 70% was used in this study. The semen was diluted at a 1:5 ratio and stored at 5 °C for a period of 72 hours. Diluted semen was evaluated at 0, 24, 48, and 72 hours, to observe its motility, kinematics, viability, plasma membrane integrity, acrosome integrity, mitochondrial activity, and semen pH. Following a 72-hour storage period, malondialdehyde (MDA) and intracellular calcium ion (Ca^{2+}) concentrations were analyzed.

Variables Measured

Sperm motility and kinematics. The analysis of sperm motility and kinematics was conducted using Computer-Assisted Sperm Analysis (CASA) (IVOS II, Hamilton Thorne). The observed motility parameters included total, progressive, slow, and static motility. Kinematic parameters were also measured, including (1) VAP (average path velocity), the average speed along the sperm path (μm/second), (2) VCL (curvilinear velocity), which represents the average speed at each point along the path passed by sperm (μm/second), (3) VSL (straight linear velocity), representing the average speed in a straight line between start to endpoints (μm/second), and (4) STR (straightness), indicating the estimated percentage of the closeness of the sperm path to a straight line (%). Additionally, the following parameters were considered: (5) LIN (linearity), representing the estimated percentage of the actual sperm path with a straight line (%), (6) WOB (wobble), indicating the estimated percentage of the distance between the actual path and the average path (%), (7) ALH (amplitude of lateral head displacement), measuring the average

Table 1. Composition of the semen diluent used

Composition	Sorbitol concentration			
	0%	1%	2%	3%
RL-EY (mL)	10	9.9	9.8	9.7
Sorbitol (g)	-	0.1	0.2	0.3
Penicillin (IU/mL)	1000	1000	1000	1000
Streptomycin (mg/mL)	1	1	1	1
Osmolality (mOsmol/kg)	268	330	377	445

Note: RL-EY (supernatant from a mixture of 90% Ringer's lactate with 10% egg yolk), Diluent pH adjusted to 7.8.

width of the oscillation head between the average path and the actual path during sperm motility (μm), and (8) BCF (beat cross frequency), quantifying the frequency of the sperm head crossing the average path in both directions (Hz).

Viability. The viability of sperm was evaluated through the utilization of eosin nigrosine staining (Agarwal *et al.*, 2016). A drop of semen was mixed with eosin nigrosine dye, homogenized, and then applied in a thin layer to a glass slide, where it was allowed to dry. The viability of 200 sperm was observed by examining them under a light microscope (Olympus, CX23, Japan) at 400x magnification, with the distinction being made between live (unstained) and dead (stained).

Plasma membrane integrity. The Hypo-osmotic Swelling Test (HOST) method was employed to examine the integrity of the sperm plasma membrane. The HOST solution was prepared by dissolving 0.09 g fructose and 0.049 sodium citrate in 10 mL of distilled water. Approximately 10 μL of semen was combined with 100 μL solution and the mixture was incubated at 37 °C for 30 minutes (Najafi *et al.*, 2019). The solution was mixed with a little eosin nigrosine before being smeared on a slide and dried. Subsequently, about 200 sperms were observed under a light microscope at 400x magnification to determine whether they had intact plasma membrane (bending in the tail, middle, or head parts) or incomplete plasma membrane (no bending).

Acrosome integrity. The semen was fixed in a 1:1 ratio with 5% formalin, subsequently smeared on a slide, and allowed to dry. The fixed preparations were incubated in a 5% formalin solution at 37 °C for 30 minutes, then rinsed with water and allowed to dry. The integrity of acrosome was observed using Coomassie brilliant blue (CBB) staining solution, as previously described by Silyukova *et al*. (2022). The solution comprised 0.25% CBB R 250 (BBI Life Sciences, Canada), 10% glacial acetic acid, and 25% methanol. Following fixation, the semen was transferred to a staining jar containing CBB solution for a period of 5 minutes at room temperature. Thereafter, it was rinsed with water and dried. The preparations were observed under a light microscope at 1000X magnification (using immersion oil) over 5-6 fields of view to determine whether sperm had intact or non-intact acrosome.

DNA damage. The presence of DNA damage was identified through the utilization of the Toluidine Blue (TB) staining method as previously described by Rui *et al*. (2017). Semen was smeared in a thin layer on a slide, allowed to dry, and then fixed in a solution of 96% ethanol and acetone in a 1:1 ratio at 4 °C for 30 minutes. Subsequently, the fixed preparations were air-dried and hydrolyzed in 0.1 N HCl solution at 4 °C for a period of five minutes. Following three 2-minute washes, the preparations were dried and stained with TB (Merck KGaA, Germany) for 20 minutes at room temperature. Approximately 200 sperm were observed under a light microscope at 400x magnification to determine whether their DNA intact (appearing as light blue) or damaged (appearing as dark blue).

Mitochondrial activity. Mitochondrial activity was observed using 3,3′-diaminobenzidine (DAB) dye solution under minimal light circumstances (Rui *et al.*, 2017). The solution was prepared by dissolving 1 mg of 3,3′-Diaminobenzidine (Sigma-Aldrich, US) in each ml of Phosphate-buffered saline (PBS). The semen was diluted with DAB solution in a 1:1 ratio and incubated at 37 °C for one hour. Subsequently, the preparations were smeared on a slide and allowed to air-dried. Approximately 100 sperm were observed under a light microscope with 1000x magnification (using immersion oil). The sperm were classified into four categories, designated as DAB I (100% active mitochondria), DAB II (>50% active mitochondria), DAB III (<50% active mitochondria), and DAB IV (inactive mitochondria).

Semen pH. The pH of the semen was determined by the use of special pH indicator paper (MQuant, Germany), which was dipped into the semen and then removed rapidly. After a brief observation period, color changes were noted and subsequently compared with a color map.

MDA. MDA concentrations were quantified utilizing a spectrophotometer at a wavelength of 532 nm as previously described by Eslami *et al*. (2016). A 250 μL sample of semen was combined with MDA solution (625 μL of 40% trichloroacetic acid, 100 μL of 1 N HCl, 50 μL of 1% Na-thiobarbiturates, and 975 μL of distilled water), heated to 100 °C for 30 minutes, and then centrifuged at 10,000 rpm for 10 minutes. In this study, the supernatant was collected and diluted to 3 mL with distilled water. The resulting solution was then analyzed for its absorbance using a spectrophotometer (Shimadzu UV-1800, Japan).

Intracellular Ca^{2+}. The sample preparation procedure for intracellular calcium analysis was based on the method described by Khaeruddin *et al*. (2024b). Approximately 50 μL of semen was taken and diluted in 150 μL of PBS, after which the solution was subjected to centrifugation at 6000 rpm for two minutes. Subsequently, the supernatant was discarded and the pellet was incubated with 10 μL of 40 μM Fluo-3 (Sigma-Aldrich, US) at room temperature and in the dark for 30 minutes. The mixture was subjected to three rounds of centrifugation at 6000 rpm for a duration of two minutes each, with the objective of washing it with PBS. The pellet was resuspended in 30 μL of PBS, placed on a coverslip, and examined for intracellular calcium concentrations using a confocal laser scanning microscope (Olympus, FV1000, Japan).

Data Analysis

A completely randomized design with four treatments and 10 replications was employed to investigate sperm motility, kinematics, viability, plasma membrane integrity, acrosome integrity, and semen pH. The data

from each storage time point were subjected to Analysis of Variance (ANOVA) followed by Duncan's multiple range test for significant p-values ($p<0.05$). The data on DNA damage and mitochondrial activity at each storage time were analyzed using the independent sample T-test with 10 replications. The data on MDA and intracellular Ca^{2+} values were subjected to analysis of ANOVA, and the statistical analysis was carried out using IBM SPSS Statistics 25 software.

RESULTS

Sperm Motility

The results showed that adding sorbitol to egg yolk Ringer lactate diluent had no effect on the motility of Gaga roosters' sperm at 0 and 24 hours of cold storage. However, a notable effect was observed after 48 and 72 hours (Table 2). At 48 and 72 hours, the addition of 1%-3% sorbitol resulted in a higher level of sperm motility compared to the control group. The results showed no significant difference in progressive motility between the various treatments at 0, 24, and 72 hours of storage intervals. However, at the 48 hours of storage, a notable divergence was observed. Furthermore, it was also observed that a solution of 2% sorbitol was more effective than the control in maintaining progressive motility. In this study, the percentage of static sperm did not differ between treatments at 0, 24, and 48 hours of storage. However, at 72 hours, the control treatment exhibited a higher percentage of static sperm than the 2% sorbitol treatment. The addition of various concentrations of sorbitol had no effect on slow motility at any storage temperature.

Sperm Kinematics

The addition of varying concentrations of sorbitol had no statistically significant impact on the majority of kinematic parameters of Gaga roosters' sperm during cold storage, except for STR (Table 3). The addition of 1-3% sorbitol resulted in a higher STR ($p<0.05$) compared to the control at 0 hours of storage.

Sperm Viability

The results showed that adding sorbitol to Ringer lactate egg yolk diluent had no effect on the viability of Gaga roosters' sperm at 0 and 24 hours of storage. However, at 48 and 72 hours of storage, there was a notable effect ($p<0.05$). The addition of sorbitol at concentrations of 1%-3% in the diluent resulted in higher viability than the control after 48 hours. At 72 hours of storage, the 2% sorbitol concentration demonstrated the highest sperm viability compared to the other treatments (Table 4).

Sperm Plasma Membrane Integrity

The results showed that the addition of sorbitol to Ringer lactate egg yolk diluent had no effect on the integrity of the plasma membrane of Gaga roosters' sperm at 0 and 24 hours of storage but had a significant effect ($p<0.05$) at 48 and 72 hours of storage (Table 4). The addition of sorbitol concentrations of 1%-3% resulted in a higher plasma membrane integrity compared to the control.

Sperm Acrosome Integrity

The results showed that the addition of sorbitol to Ringer lactate egg yolk diluent had no statistically significant impact on the acrosomal integrity of Gaga roosters' sperm (Table 4). Integrity of the acrosome in the rooster sperm head (red circle) with Coomassie brilliant blue staining can be seen in Figure 1.

Table 2. Motility of Gaga rooster sperm during cold storage with different concentrations of sorbitol in the diluent

Storage time (h)	Variables	Sorbitol concentration in the diluent			
		0%	1%	2%	3%
0	Motility (%)	88.32±2.43	87.90±1.84	90.36±1.82	90.14±2.38
24		74.22±4.35	75.22±3.75	78.09±3.22	76.53±3.57
48		52.96±3.30^{a}	63.03±4.21^{ab}	67.89±3.87^{b}	66.86±4.45^{b}
72		35.61±4.57^{a}	46.26±3.26^{b}	48.88±2.23^{b}	47.03±3.48^{b}
0	Progressive (%)	47.02±4.82	54.32±3.97	56.61±3.08	57.29±4.39
24		11.01±1.59	12.42±1.77	12.11±1.00	12.36±1.44
48		3.82±0.92^{a}	4.07±0.64^{ab}	6.50±0.83^{b}	6.28±0.90^{ab}
72		0.34±0.09	0.36±0.10	0.63±0.18	0.65±0.21
0	Static (%)	11.64±2.43	12.10±1.84	9.64±1.82	9.86±2.38
24		25.78±4.35	24.78±3.75	21.90±3.22	23.47±3.57
48		41.64±7.30	36.97±4.21	32.11±3.87	33.14±4.45
72		64.39±4.57^{a}	54.24±3.21^{ab}	51.12±2.23^{b}	50.97±4.35^{b}
0	Slow (%)	14.59±3.03	9.61±1.51	9.47±1.11	9.98±1.26
24		28.65±3.40	27.47±3.50	21.49±4.03	33.45±2.71
48		30.81±5.66	39.64±4.33	33.42±3.26	40.9±2.05
72		28.12±4.45	33.98±3.81	27.47±2.54	31.68±4.14

Note: Different letters on the same row indicate significant differences ($p<0.05$).

Table 3. Kinematic of Gaga rooster sperm during cold storage with different concentrations of sorbitol in the diluent

Storage time (h)	Variables	Sorbitol concentration in the diluent			
		0%	1%	2%	3%
0	VAP (μm/s)	60.19±4.54	65.23±3.58	66.56±2.47	67.68±4.22
24		29.45±2.01	29.74±1.75	29.62±1.21	27.86±1.24
48		19.54±1.45	19.99±1.17	23.21±1.31	21.57±1.19
72		13.17±1.73	12.07±0.92	13.87±0.86	15.07±1.59
0	VSL (μm/s)	49.12±3.98	53.33±3.09	56.35±2.20	57.62±4.28
24		22.02±1.35	22.59±1.88	23.03±1.54	21.36±1.52
48		14.46.1.24	14.25±0.67	17.09±1.27	15.87±0.97
72		9.71±1.63	8.02±0.67	10.15±0.86	12.12±1.75
0	VCL (μm/s)	95.79±5.89	102.48±4.48	100.99±3.82	103.76±4.93
24		56.07±4.01	52.52±3.54	54.19±2.20	50.92±1.27
48		40.39±3.09	40.48±2.22	45.62±1.67	42.27±2.41
72		28.50±2.63	28.19±2.34	30.40±1.87	28.79±2.25
0	STR (%)	79.90±1.14^{a}	80.68±0.78^{ab}	83.63±0.51^{b}	83.69±1.47^{b}
24		73.17±1.93	73.79±2.37	75.89±3.32	74.47±2.38
48		72.87±2.73	71.80±2.17	72.49±1.59	72.49±1.53
72		70.66±4.32	68.63±4.50	73.80±3.21	78.12±3.99
0	LIN (%)	47.20±1.76	50.02±1.31	52.56±0.87	52.38±1.92
24		39.75±2.72	39.82±3.43	43.99±4.01	41.01±3.01
48		38.16±3.02	38.29±2.08	38.50±2.71	38.79±1.97
72		40.69±5.68	35.56±3.91	37.85±3.57	46.16±5.38
0	ALH (μm)	5.04±0.28	5.20±0.20	5.17±0.24	5.43±0.23
24		3.66±0.31	3.43±0.20	3.35±0.26	3.21±0.15
48		2.55±0.24	2.89±0.22	2.91±0.13	2.85±0.23
72		1.80±0.17	1.95±0.22	2.03±0.20	1.78±0.21
0	BCF (Hz)	28.84±0.49	29.20±0.33	27.88±0.70	28.05±0.91
24		31.23±0.51	31.34±0.58	30.05±0.37	30.31±0.64
48		31.79±0.37	32.30±0.70	32.38±1.17	31.05±0.96
72		36.70±1.96	35.47±1.47	33.55±0.87	33.89±0.81
0	WOB (%)	59.47±1.59	61.88±0.97	63.54±0.89	62.80±1.38
24		52.14±1.87	52.30±2.49	55.39±2.61	53.18±2.21
48		48.88±2.05	50.22±1.07	50.45±2.20	50.45±1.44
72		49.45±4.66	45.95±2.70	47.43±2.67	54.22±4.25

Note: Different letters on the same row indicate significant differences ($p<0.05$). VAP= average path velocity, representing the average speed along the sperm path (μm/second); VCL= curvilinear velocity, representing the average speed at each point along the path passed by sperm (μm/second); VSL= straight linear velocity, representing the average speed in a straight line between start to endpoints (μm/second); STR= straightness, indicating the estimated percentage of the closeness of the sperm path to a straight line (%); LIN= linearity, representing the estimated percentage of the actual sperm path with a straight line (%); ALH= amplitude of lateral head displacement, measuring the average width of the oscillation head between the average path and the actual path during sperm motility (μm); BCF= beat cross frequency, quantifying the frequency of the sperm head crossing the average path in both directions (Hz); WOB= wobble, indicating the estimated percentage of the distance between the actual path and the average path (%).

Table 4. Viability, membrane integrity, and acrosome integrity of Gaga rooster sperm during cold storage with different concentrations of sorbitol in the diluent

Storage time (h)	Variables	Sorbitol concentration in the diluent			
		0%	1%	2%	3%
0	Viability (%)	94.06±1.09	95.27±1.04	96.15±0.46	95.03±1.51
24		84.53±2.76	89.02±1.29	89.93±0.73	88.68±1.62
48		74.39±2.99^{a}	81.02±1.88^{b}	83.61±1.74^{b}	80.65±1.66^{b}
72		63.02±3.67^{a}	69.60±2.90^{ab}	75.25±2.50^{b}	72.24±3.08^{ab}
0	Membrane integrity (%)	94.60±0.94	96.07±1.06	97.16±0.54	95.81±0.93
24		86.48±1.06	89.75±1.26	90.26±0.81	90.04±1.57
48		75.85±1.95^{a}	80.47±2.09^{ab}	82.90±1.31^{b}	81.90±1.72^{b}
72		64.66±1.85^{a}	71.52±2.45^{b}	76.84±1.39^{b}	73.45±1.62^{b}
0	Acrosome integrity (%)	98.63±0.34	99.34±0.27	99.22±0.25	98.70±0.33
24		97.18±0.37	97.44±0.52	97.62±0.41	96.69±0.45
48		96.11±0.48	96.04±0.48	96.38±0.57	95.27±0.61
72		94.23±0.63	94.85±0.54	95.14±0.60	93.56±0.65

Note: Different letters on the same row indicate significant differences ($p<0.05$).

Sperm DNA Damage

The results showed that the addition of 2% sorbitol to Ringer lactate egg yolk diluent had no statistically significant impact on sperm DNA damage during storage (Figures 2 and 3).

Sperm Mitochondrial Activity

The results showed that the addition of 2% sorbitol to Ringer lactate egg yolk diluent had no impact on the mitochondrial activity (Figure 4) of Gaga roosters' sperm (Table 5).

Semen pH

The results showed that the pH of Gaga roosters' semen did not differ significantly ($p>0.05$) across varying concentrations of sorbitol (Figure 5).

MDA

The concentration of MDA increased significantly ($p<0.05$) from 1.82 µM in fresh semen to 4.85-5.33 µM after 72 hours of cooling (Table 6). No significant variation was observed in MDA levels between samples with and without 2% sorbitol.

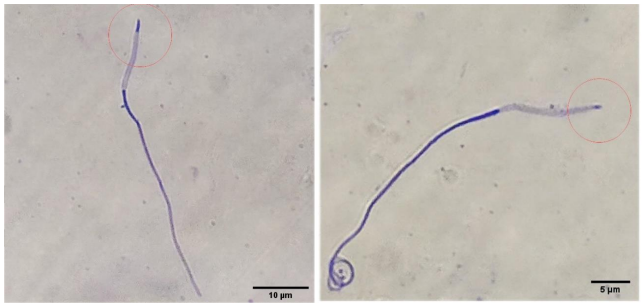

Figure 1. Integrity of the acrosome in the Gaga rooster sperm head (red circle) with coomassie brilliant blue staining, where the intact acrosome stained with blue is conical in shape (left) and pointed while the damaged acrosome appears shortened and blunt (right).

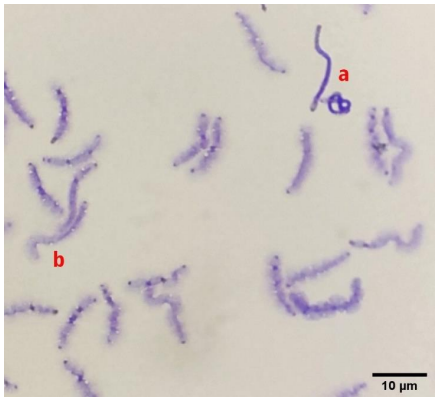

Figure 3. DNA integrity in Gaga rooster sperm with Toluidine blue (TB) staining, where damaged DNA is stained dark blue (a) and intact DNA is stained light blue (b).

Intracellular Ca^{2+} Concentrations

The results showed that the treatment had a statistically significant impact on intracellular Ca^{2+} concentrations ($p<0.05$) (Table 6, Figure 6). The concentrations in fresh semen and liquid semen with 2% sorbitol diluent which were stored for 72 hours, exhibited a notable degree of similarity with values of 136.16 au and 134.63 au, respectively. Conversely, intracellular calcium concentrations in liquid semen held for 72 hours in the absence of sorbitol were lower at 12.99 au.

DISCUSSION

The quality of sperm was a critical factor in the success of the artificial insemination procedure. The presence of high-quality sperm, exhibiting optimal motility and viability, markedly elevates the probability of successful fertilization. Sperm with optimal motility exhibited the capacity to swim efficiently toward the egg. Sun *et al.* (2019) reported that sperm motility values

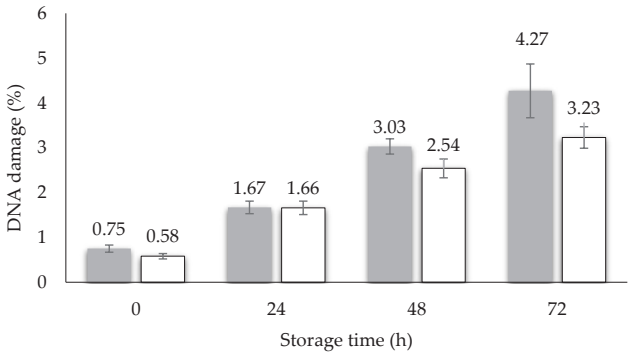

Figure 2. DNA damage in Gaga rooster sperm during chilled storage with the addition of 2% sorbitol in the diluent. ■ Control □ Sorbitol 2%

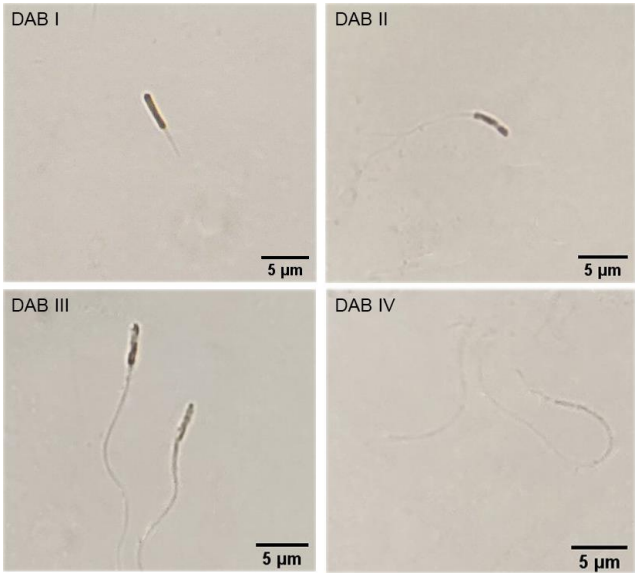

Figure 4. Mitochondrial activity in Gaga rooster sperm with DAB staining, which is classified into 4: DAB I: All active mitochondria, DAB II: >50% active mitochondria, DAB III: <50% active mitochondria, DAB IV: inactive mitochondria.

Table 5. Mitochondrial activity of Gaga rooster sperm during cold storage with different concentrations of sorbitol in the diluent

Storage time (h)	Treatments	Percentage of mitochondrial activity			
		DAB I (%)	DAB II (%)	DAB III (%)	DAB IV (%)
0	Control	80.38±1.05	14.54±1.19	3.88±0.65	1.19±0.19
	Sorbitol 2%	84.34±1.03	12.81±1.01	1.69±0.24	1.17±0.13
24	Control	66.70±3.06	23.26±2.56	8.57±1.16	1.47±0.27
	Sorbitol 2%	72.31±3.72	19.46±3.35	6.86±0.71	1.38±0.17
48	Control	59.12±3.33	26.90±2.79	12.00±1.13	1.98±0.28
	Sorbitol 2%	63.68±3.09	25.36±3.18	8.97±0.54	1.98±0.18
72	Control	42.55±2.82	37.52±3.22	17.15±1.68	2.78±0.40
	Sorbitol 2%	54.14±3.32	29.48±2.48	13.69±1.81	2.69±0.27

Note: DAB I= 100% active mitochondria, DAB II= >50% active mitochondria, DAB III= <50% active mitochondria, and DAB IV= inactive mitochondria.

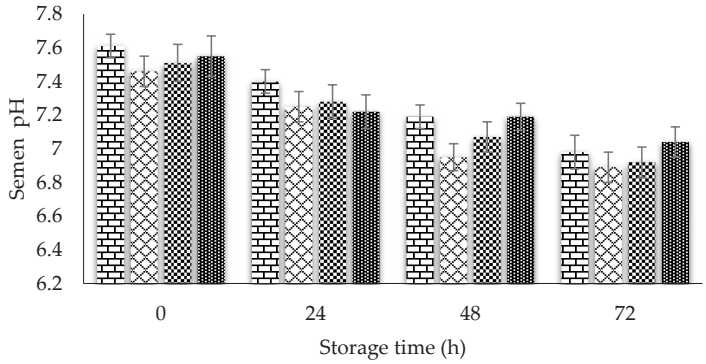

Figure 5. pH of Gaga rooster semen during chilled storage with different concentrations of sorbitol in the diluent. Control Sorbitol 1% Sorbitol 2% Sorbitol 3%

Table 6. Intracellular Ca2+ and malondialdehyde (MDA) concentrations of Gaga rooster semen

Variables	Fresh Semen	Semen stored for 72 hours (control)	Semen stored for 72 hours (sorbitol 2%)
MDA (μM)	1.82±0.20[a]	5.33±1.11[b]	4.85±0.95[b]
Intracellular Ca^{2+} (au)	137.79±6.30[a]	121.99±3.14[b]	134.63±2.13[a]

Note: Different letters on the same row indicate significant differences ($p<0.05$).

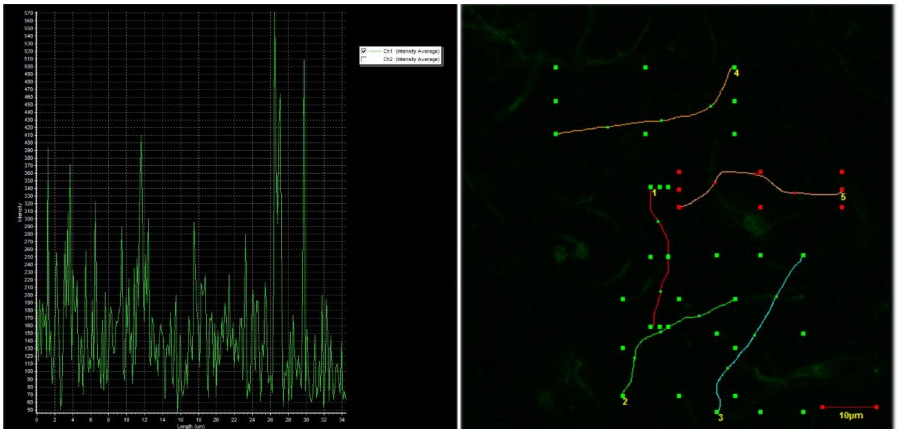

Figure 6. Measurement of Ca2+ in rooster sperm using Fluo-3 staining analyzed using CLSM. Left image: Ca2+ intensity, right image: DIC + fluorescence.

in chickens are similar to fertility values. The report showed that low motility, at 38.85% with viability at 60.33%, could produce fertility at 38.93%, while motility at 67.84% with viability at 71.17% resulted in a fertility rate of 62.36%. The integrity of sperm organelles during the preservation process was a critical factor that crucial for successful fertilization. Some organelles, such as the mitochondria, the acrosome, and the nucleus, played vital roles in energy production, egg penetration, and genetic material delivery, respectively. The preservation of the functionality and structure of these organelles, significantly enhanced the probability of successful fertilization and healthy embryo development.

The objective of this study was to examine the effect of sorbitol supplementation in roosters' semen diluent. It was of great importance to maintain the pH and os-

molality of diluted semen in order to ensure the viability of sperm during storage (Partyka & Niżański, 2022). The pH range remained suitable for sperm viability, consistent with the findings of Blesbois (2012) that roosters' sperm could tolerate a pH range of 6.0 to 8.0. As McCue (2021) observed, semen pH typically declines during storage due to metabolic processes, leading to the formation of lactic acid. The addition of sorbitol to the diluent in this study enhanced the osmolality, which is in accordance with the opinion of Ip & Medzhitov (2015) that sorbitol enhanced media osmolality. The osmolality of the diluent containing 1%-3% sorbitol was found to be compatible with roosters' sperm, in accordance with the findings of Sarkar (2020), which indicated that rooster sperm could survive at an osmolality between 250 to 450 mOsm/kg. Sitaula *et al*. (2010) reported that sorbitol acted as an excellent osmolyte during sperm dehydration, thereby maintaining sperm motility.

The motility of sperm is an important indicator of the quality and fertility of roosters' semen (Sun *et al*., 2019). The addition of sorbitol to the diluent was observed to enhance total motility, which is likely attributable to its function as an alternative energy source. According to Cao *et al*. (2009), sorbitol plays a role in maintaining sperm motility during incubation by participating in fructose synthesis through the polyol pathway. Subsequently, the fructose was digested by glycolysis, resulting in the generation of pyruvate, adenosine triphosphate (ATP), which serve as the energy source. The electron transport chain in mitochondria and oxygen consumption were identified as the primary components of mitochondrial respiration that are closely associated with ATP synthesis (Sushadi *et al*., 2023). The glycolysis process contributed hydrogen groups to NAD^+, resulting in the production of NADH (Kawanami *et al*., 2016), which was subsequently utilized by NADH dehydrogenase complex-1 in the electron transport chain to generate ATP (Walker & Tian, 2018). The present study, demonstrated a decline in sperm motility in Gaga roosters following semen storage. Słowińska *et al*. (2018) observed a reduction in mitochondrial membrane potential and ATP production during storage, which resulted in a decline in sperm motility.

In this study, the total motility of sperm stored for 24 hours was higher than that observed in previous reviews. These previous reviews used Lake diluent 42.1%-61.3% (Masoudi *et al*., 2019) and 75% (Blank *et al*., 2021), as well as 68.5% NaCl diluent (Chankitisakul *et al*., 2022). Similarly, the motility stored for 48 hours was also higher than that observed when using egg yolk Ringer lactate diluent (33.5%-41.5%) (Arif *et al*., 2023), Lake diluent (8.7%-22.5%) (Masoudi *et al*., 2019), and BPSE diluent (3.7%-28.2%) (Fattah *et al*., 2017). After 0 hours of storage, progressive motility was higher than that reported in previous reviews by Alipour-Jenaghard *et al*. (2023) (38.4%-40.3% using Lake diluent), Suwimonteerabutr *et al*. (2024) (17.8% using BPSE diluent), and Khodaei-Motlag *et al*. (2022) (38.2%-42.8% using Lake diluent). Conversely, 24 hours of storage period resulted in a slight reduction in progressive motility compared to the findings of Suwimonteerabutr *et al*. (2024) (14.8%) and Alipour-Jenaghard *et al*. (2023) (15%-22.5%). The progressive motility at 48 hours was comparable to that reported by Masoudi *et al*. (2020) (2.8-10.6%) and Masoudi *et al*. (2019) (3.1-6%) using Lake diluent.

Several sperm kinematic parameters in this study were similar to the results of previous studies. Suwimonteerabutr *et al*. (2024) found that the VCL and ALH at 24 hours, the VSL at 48 hours, and the WOB at 72 hours exhibited minimal variation. These values were 55.8 μm/s, 3.62 μm, 14.9 μm/s, and 47.3%, respectively, in BPSE diluent. The VCL, VSL, LIN, and ALH at 24 hours of storage in the present study were identical to those reported by Łukaszewicz *et al*. (2020). The values for these parameters were 47.8-55.9 μm/s, 20.1-21.8 μm/s, 37.4-40.7 %, and 3.2-3.4 μm, respectively, and they were obtained using EK diluent.

The addition of sorbitol was found to preserve sperm viability, which depends on energy metabolism to survive (Reynolds *et al*., 2017). The addition of sorbitol provided enhanced protection by facilitating the penetration of the plasma membrane (Sitaula *et al*., 2010) and acting as an energy source (Cao *et al*., 2009). It was observed that semen storage had an adverse effect on sperm viability, which explained the observed reduction in sperm motility. As stated by Tesfay *et al*. (2020) there is a positive correlation between sperm viability and sperm motility in roosters. The reduction in sperm viability observed after 48 hours of semen collection was attributed to significant alterations in the lipid composition of the plasma membrane (Partyka, & Niżański, 2022). Parodi (2014) observed that a reduction in sperm viability is associated with an increase in reactive oxygen species (ROS) generated during metabolism.

The viability of sperm stored for 24 hours in the present study was comparable to the findings of Partyka *et al*. (2015), who reported sperm viability rates of 89.8%-92.8% using an EK diluent. This result exceeded previous reviews using Lake diluent, which demonstrated sperm viability of 48.9%-65.2% (Masoudi *et al*., 2019) and 54%-60.8% (Khodaei-Motlagh *et al*., 2022), phosphate buffer diluents obtaining viability of 82.53% (Eslami *et al*., 2016), 79.67%-84.75% (Eslami *et al*., 2018), and 82.66% (Rad *et al*., 2016). Additionally, EK diluent achieved a sperm viability of 79.2% (Łukaszewicz *et al*., 2020). Furthermore, the viability of sperm at 48 hours of storage was also higher than that reported in numerous previous investigations, which indicated 12.5%-28.4% (Masoudi *et al*., 2019) and 22.7%-28.6% (Khodaei-Motlagh *et al*., 2022) with Lake diluent, and 72.80% (Eslami *et al*., 2016), 62.81%-68.87% (Eslami *et al*., 2018), and 70.60% (Rad *et al*., 2016) with phosphate buffer diluent.

The addition of sorbitol in the diluent was observed effectively maintain the integrity of sperm plasma membrane, thereby indicating the ability of this polyol to protect the membrane from damage. Sootsuwan *et al*. (2013) reported that such a polyol may contribute to the maintenance of the membrane bilayer, increase membrane fluidity, and facilitate cell recovery from stress. It was observed that storage could reduce the integrity of sperm plasma membrane due to the presence of polyunsaturated fatty acids (PUFAs) (Juan *et al*., 2021), which

were susceptible to free radical damage, resulting in weakening membrane integrity (Yadav *et al.*, 2019).

The plasma membrane integrity observed over the 24-hour period was within the range reported by Thananurak *et al*. (2019), which was 77.96%-93.25% using BHSV diluent with additional sucrose. These findings surpassed those of previous studies utilizing BPSE diluent, which showed 45.1%-70.3% (Fattah *et al.*, 2017) and 71.79%-75.98% (Sharideh *et al.*, 2019), as well as Lake diluent, with exhibited a notable range of 50.8%-70.2% (Masoudi *et al.*, 2020). Furthermore, the plasma membrane integrity after 48 hours of storage was also higher than in previous reviews. In particular, Ringer's lactate egg yolk diluent reported a range of 73.75%-79.25% (Arif *et al.*, 2023), while Lake diluent showed a range of 17.8%-31.5% (Masoudi *et al.*, 2019) and 27.5%-32.1% (Masoudi *et al.*, 2020). Additionally, sperm in BPSE diluent reported a range of 11.5%-35.6% (Fattah *et al.*, 2017).

The acrosome contains enzymes that facilitate the penetration of the egg by sperm during the process of fertilization. The present study demonstrated that storage resulted in a slight decline in the structural integrity of acrosome cap. The findings were consistent with the hypothesis proposed by Blank *et al*. (2021), which suggested that the acrosome of roosters' sperm remained intact when stored at 5 °C, but underwent destruction when stored at 37 °C. The results of this analysis were more favorable than those of Lemoine *et al*. (2011), who observed a significant decline in the percentage of intact roosters' sperm acrosome after 48 hours of storage at 4 °C. Furthermore, storage may result in a minor increase in DNA damage due to an imbalance of free radicals and antioxidants, leading to lipid peroxidation (Opuwari & Henkel, 2016; Noegroho *et al*., 2022).

This study found that the percentage of acrosome integrity at 24 hours of storage was higher than in the majority of other investigations. For example, Tvrdá *et al*. (2023) reported a value of 81.91% using EM diluent, while Mavi *et al*. (2022) reported values between 68.33%-78.04% using egg yolk plasma diluent. Furthermore, the percentage of intact acrosome at 48 and 72 hours was also higher than that reported by Mavi *et al*. (2022), who observed 52.21%-74.60% at 48 hours and 47.33%-69.19% at 72 hours using egg yolk plasma diluent. Balogun (2021) reported that the acrosome integrity of rooster's sperm remained consistently intact for up to 72 hours when stored in Tris' coconut water-orange juice diluent.

The DNA damage observed after 0 and 48 hours in the present study was comparable to that reported by Farid *et al*. (2021), which was 0.87% and 2%-2.62% respectively, using Ringer lactate egg yolk diluent. The level of sperm DNA damage was found to be even lower than that observed in previous studies. Previous studies had reported levels of 7.55%-13.55% at 48 hours of storage using Phosphate egg yolk diluent (Bebas *et al.*, 2015) and 7.75%-10.78% at 72 hours using Ringer lactate egg yolk diluent (Khaeruddin *et al.*, 2024a).

Mitochondria, the organelles responsible for sperm respiration, played a crucial role in energy production, which was required for sperm progression. This progression necessitated a corresponding enhancement in mitochondrial respiratory capacity, thereby underscoring the vital role of mitochondrial oxidative phosphorylation in sperm motility (Sangani *et al.*, 2017). In the present study, storage was observed to result in a slight decrease in mitochondrial activity. As posited by Blank *et al*. (2021), prolonged storage periods have been demonstrated to exert a deleterious effect on mitochondrial activity and sperm motility. Additionally, it was observed that liquid storage resulted in abnormally elevated levels of reactive oxygen species (ROS), which caused oxidative damage to mitochondria and accelerated mitochondria-dependent apoptosis (Liu *et al.*, 2019).

This present study found a higher proportion of sperm exhibiting active mitochondria (DAB I) at 24 hours of storage compared to previous investigations using Lake diluent. These previous studies reported a range of 55.4%-69.7% (Masoudi *et al.*, 2019), 55%-61.3% (Khodaei-Motlagh *et al.*, 2022), and 50.9%-62.1% (Alipour-Jenaghard *et al.*, 2023). However, this value was lower than that reported by Kheawkanha *et al*. (2023), who observed mitochondrial activity in 77.85%-83.69% of sperm using IGGKPh diluent. Furthermore, mitochondrial activity during 48 hours of storage period was also higher than that observed in previous reports using Lake diluent, which indicated 19.6%-26.5% (Khodaei-Motlagh *et al.*, 2022), 14.5%-17.8% (Masoudi *et al.*, 2019), and 16.5%-20.4% (Alipour-Jenaghard *et al.*, 2023).

A slight decrease in semen pH was observed from 0 to 72 hours of storage with pH values ranging from 7.46 to 7.61 to 6.89 to 7.04. These results are consistent with those of Sushadi *et al*. (2023), who observed a decline in the pH of roosters semen with BPSE diluent from 7.02-7.03 at 0 hours to 6.44-6.56 after 72 hours.

The process of lipid peroxidation resulted in the production of harmful metabolites, including MDA and 4-hydroxynonenal (HNE). These metabolites disrupted the integrity of plasma membrane, thereby reducing sperm quality and fertilization ability (Aitken *et al.*, 2016). The findings of this study indicated that semen storage for 72 hours may result in an increase in MDA concentration and concomitant decrease in plasma membrane integrity. Free radicals and ROS initiated a chain reaction of lipid peroxidation by attacking PUFAs (Prochowska *et al.*, 2024). The sperm cell membrane of roosters, which contained high levels of PUFAs, was particularly susceptible to lipid peroxidation (Mussa *et al.*, 2021). The action of free radicals on unsaturated lipid chains, leading to the formation of hydro peroxidized lipids and alkyl radicals, which in turn alter the structure, fluidity, and integrity of the membrane (Yadav *et al.*, 2019).

The present study found that the MDA concentrations during 72 hours of storage periods were higher than those reported by Kheawkanha *et al*. (2023) (4.15 μM with IGGKPh diluent) and Suwimonteerabutr *et al*. (2024) (3.58 μM using BPSE diluent). The value was lower than those reported by Alipour-Jenaghard *et al*. (2023) (5.55-6.72 μM with Lake diluent), Masoudi *et al*. (2020) (5.10-6.82 μM with Lake diluent after 48 hours of storage), as well as Khodaei-Motlagh *et al*. (2022) (5.73-6.50 μM with Lake diluent at 45 hours).

Calcium (Ca^{2+}) plays an essential role in numerous cellular activities and is maintained at lower concentrations within cells than in external environments to prevent adverse effects (Matuz-Marez *et al.,* 2022). The regulation of intracellular Ca^{2+} had an effect on sperm motility and acrosome reactions, both of which were necessary for egg penetration (Sushadi *et al.,* 2023). The maintenance of Ca^{2+} homeostasis was found to be essential for the preservation of sperm motility *in vitro* (Froman, 2016). Intracellular Ca^{2+} is necessary for the maintenance of avian sperm motility (Ashizawa *et al.,* 1992) and also plays a pro-vital role in signaling networks that regulate energy consumption (Sushadi *et al.,* 2023). The addition of exogenous Ca^{2+} can increase the concentration of free intracellular Ca^{2+}, which can restore rooster sperm motility (Nguyen *et al.,* 2016). An increase in bicarbonate and Ca^{2+} levels results in the activation of intracellular pathways associated with sperm mobility. The process commences with the activation of adenyl cyclase, which results in an increase in cAMP and subsequently the activation of protein kinase A (PKA). Furthermore, an increase in Ca^{2+} levels activates CaM, which in turn activates CaMK and AMP (Nguyen, 2019).

The findings of this study indicated that the inclusion of sorbitol in the diluent maintained intracellular calcium concentration stability for up to 72 hours of storage. Conversely, the absence of the compound resulted in a decline in intracellular calcium concentration. The Ca^{2+}-ATPase enzyme on the plasma membrane plays a pro-vital role in regulating cellular Ca^{2+} homeostasis (Gong *et al.,* 2018). However, cooling resulted in membrane hydrolysis and damaged to the Ca^{2+}-ATPase (Sieme *et al.,* 2015; Jin & Yang, 2017). In accordance with the observations made by Yoo (2014), sorbitol was found to maintain the activity of Ca^{2+}-ATPase in frozen surimi, thereby indicating that the compound played a role in preserving the enzyme's functionality in semen diluents during the storage periods.

CONCLUSION

In conclusion, the addition of sorbitol to the RLEY diluent was found to significantly maintain the quality of Gaga roosters' sperm during cold storage for 72 hours and can be used for artificial insemination. Furthermore, the addition of 1%-2% sorbitol led to a notable enhancement in the total and progressive motility, viability, plasma membrane integrity, and intracellular calcium concentrations of the sperm.

CONFLICT OF INTEREST

The authors of this article declare that there are no conflicts of interest among them.

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