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Full Length Article

Effects of Sorbitol and Butylated Hydroxytoluene on Quality, Lipid Peroxidation and Intracellular Calcium Concentration of Gaga Chicken Frozen Sperm

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Abstract

Gaga chicken is a native Indonesian breed, requiring preservation through sperm cryopreservation. In this process, the addition of sugar in sperm diluent serves as a cryoprotectant, and the incorporation of antioxidants prevents lipid peroxidation in sperm. Therefore, this study aimed to investigate the effect of adding sorbitol, butylated hydroxytoluene (BHT), and the combination in sperm diluent on sperm quality, lipid peroxidation, and intracellular calcium (Ca^{2+}) concentration in Gaga chicken frozen sperm. Pooled sperm was collected from male Gaga chickens and divided into 4 tubes. Each tube represented a different treatment, containing Ringer's lactate egg yolk (RLEY) diluent alone, RLEY with 3 mM BHT, RLEY with 2% sorbitol, and RLEY with 3 mM BHT and 2% sorbitol. Sperm was put in mini straws, equilibrated, pre-freezed, and stored frozen. Motility, kinematics, viability, sperm Ca^{2+} concentration, and malondialdehyde (MDA) concentration were measured post-thawing. The results showed that treatment had a significant effect ($P < 0.05$) on sperm motility, various kinematic parameters, viability, Ca^{2+} concentration, and post-thawing MDA concentration. In post-thawing, progressive motility and viability were highest in P2 and P3 diluents, while the lowest was observed in Ca^{2+} and MDA concentrations. The addition of sorbitol in the diluent produced the best sperm quality, maintaining intracellular Ca^{2+} concentration and preventing lipid peroxidation in Gaga chicken frozen sperm.

Keywords: Butylated hydroxytoluene; Intracellular calcium; Chicken sperm; Lipid peroxidation; Sorbitol

Introduction

Indonesia is home to several local chickens used as a food source or ornamental animals. Gaga chicken is a native Indonesian breed characterized by a unique voice with a longer sound duration compared to others. This sound is similar to laughing, making it a sought-after ornamental chicken, thereby increasing the selling price, which varies based on sound quality. Minister of Agriculture in Decree No. 2920/Kpts/OT.140/6/2011 has decided that Gaga chicken is a wealth of livestock genetic resource in Indonesia, requiring protection and preservation (Khaeruddin *et al.* 2022).

The significance of Gaga chicken shows the need for implementing preservation efforts through sperm cell freezing (cryopreservation) technology. Thélie *et al.* (2019) stated that chicken sperm stored frozen for 18 years in liquid nitrogen retained the ability to fertilize egg cells, proving the efficacy in restoring chicken genetic resources. Moreover, the success of freezing depends on the type of diluent used. Although Beltsville diluent has been commonly used in various studies (Nabi *et al.* 2016; Lotfi *et al.* 2017; Siari *et al.* 2022; Getachew *et al.* 2023), Ringer's lactate supplemented with 10% egg yolk showed superiority in maintaining the quality of chicken frozen sperm (Telnoni *et al.* 2017). This diluent has been used in sperm-freezing studies of various local Indonesian chicken breeds (Telnoni *et al.* 2017; Khairuddin *et al.* 2019; Hidayat *et al.* 2022; Khaeruddin *et al.* 2022).

Cryopreservation causes a decrease in chicken sperm quality due to the formation of intracellular ice crystals, and the accumulation of reactive oxygen species (ROS) which causes oxidative stress (Leao *et al.* 2021; Masoudi *et al.* 2021; Partyka and Niżański 2021). To minimize damage caused by the freezing process, cryoprotectants are added to the diluent, with careful selection of the material for an effective poultry sperm freezing protocol (Mosca *et al.* 2020). Cryoprotectants are divided into two, namely

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penetrating capable of crossing cell membranes such as dimethylsufoxide (DMSO), and non-penetrating, which are extracellular such as polyvinyl pyrrolidone and trehalose (Murray and Gibson 2022). The head of avian spermatozoa has a smaller cytoplasmic volume, resulting in a lower ability to absorb penetrating cryoprotectant agents (Long 2013; Janosikova *et al.* 2022). Consequently, a combination of penetrating and non-penetrating cryoprotectant agents is needed in the diluent (Mosca *et al.* 2016).

The addition of sugar as a non-penetrating cryoprotectant in the diluent has been reported to be effective in maintaining the quality of cryopreserved chicken sperm (Stanishevskaya *et al.* 2021a, b). Other reports showed that sugar alcohols such as sorbitol were more effective compared to glucose or fructose in maintaining mammalian sperm quality (Pojprasath *et al.* 2011; Wu *et al.* 2016). Specifically, sorbitol also has several benefits, including an energy source for sperm (Cao *et al.* 2009), with potential as an antioxidant in cells (Kang *et al.* 2007). Despite these advantages, the effectiveness of sorbitol for chicken frozen sperm has not been reported, prompting the selection in this study.

The cell membrane structure of chicken sperm contains high levels of polyunsaturated fatty acids (Mussa *et al.* 2021), which require higher antioxidant protection due to susceptibility to lipid peroxidation (Janosikova *et al.* 2022). In this context, Butylated hydroxytoluene (BHT) is a synthetic phenolic antioxidant (Yehye *et al.* 2015) that is superior to vitamin E in preventing oxidation reactions (Gultom and Ginting 2018). As a chain-breaking antioxidant, BHT reacts with peroxyl radicals, disrupting the lipid peroxidation propagation reaction and inhibiting lipid autoxidation (Olmedo *et al.* 2019).

Several studies showed the effectiveness of BHT in maintaining frozen sperm quality from various cryopreserved mammal species (Merino *et al.* 2015; Seifi-Jamadi *et al.* 2016; Jara *et al.* 2019; Sun *et al.* 2020). However, in chicken, a concentration of 1 mM BHT was reported to negatively affect the quality of cryopreserved chicken sperm (Kumar *et al.* 2019). To address the limitation, analysis was conducted focusing on increasing BHT concentration to 3 m*M* to maintain the quality of Gaga chicken frozen sperm. Therefore, this study aimed to investigate the effect of adding sorbitol, BHT, and their combination to diluent on sperm quality, lipid peroxidation and Ca^{2+} concentration in Gaga chicken frozen sperm.

Materials and Methods

Diluent preparation

In this study, Ringer's lactate-egg yolk (RLEY) served as the primary diluent, which was prepared by mixing 10% egg yolk and 90% Ringer's lactate (PT. Widatra Bakti, Indonesia) (Khairuddin *et al.* 2019). The composition of ringer lactate used is 1.55 g sodium lactate, 3 g sodium chloride, 0.15 g potassium chloride, and 0.1 g calcium chloride in 500 mL of sterile water. Subsequently, egg yolk and Ringer's lactate were homogenized and centrifuged at 3000 rpm for 15 min. The supernatant was used as a basic diluent, where penicillin-streptomycin (PT. Meiji, Indonesia), 7% DMSO (Merck KGaA, Germany), including 3 m*M* BHT (Sigma, US) and 2% sorbitol (Merck KGaA, Germany) were added as treatment, with the respective composition shown in Table 1.

Farm management and sperm collection

This study used four male Gaga chickens aged 12 months which were kept in individual cages measuring 55 x 60 x 60 cm^3. Male chickens were given 100 g/day of commercial feed with a composition of 17% crude protein, 3% crude fat, 7% crude fiber, and 14% ash content, while drinking water was provided ad libitum. Sperm was collected three times a week using a cloacal massage technique (Kucera and Heidinger 2018), aspirated with a tuberculin syringe and taken to the laboratory for evaluation and dilution.

Cryopreservation of sperm

Pooled sperm was divided into four tubes and diluted based on treatment, with sperm count per tube of at least 200 million cells and motility of at least 70%, which were packaged in 0.25 mL ministraws (IMV, France). Sperm equilibration was carried out at a temperature of 5°C and for 2 h (Santiago-Moreno *et al.* 2011). Subsequently, pre-freezing was carried out on nitrogen vapor with a surface height of liquid nitrogen and a straw of 3 cm (Madeddu *et al.* 2016) for 10 min (Mosca *et al.* 2016). Frozen sperm was stored in containers for 24 h and thawing was carried out at a temperature of 37°C for 30 s (Shah *et al.* 2016). The cryopreservation process was conducted 10 times as replicates.

Assessment of sperm motility and kinematics

Sperm motility and kinematics were measured using Computer Assisted Sperm Analysis (IVOS II, Hamilton Thorne) on sperm before freezing and post-thawing. The motility parameters measured included total, progressive, slow, and static motility. The kinematic parameters measured are distance average path (DAP), distance straight-line (DSL), distance curve-line (DCL), velocity average pathway (VAP), velocity straight line (VSL), velocity curve linear (VCL), linearity (LIN), straightness (STR), and amplitude lateral head (ALH), beat cross frequency (BCF) and wobble (WOB).

Assessment of sperm viability

Observation of sperm viability in sperm before freezing and post-thawing was carried out following the procedures of Agarwal *et al.* (2016). In this process, sperm was dropped on a glass object followed by a drop of eosin-nigrosin stain, homogenization and smearing on another glass object.

Subsequently, 100 sperm were observed using a light microscope (Olympus CX23, Japan) with 40x magnification, where the heads of live sperm were characterized by not absorbing color and dead sperm absorbing color.

Malondialdehyde concentration

MDA was measured in post-thawing sperm using the thiobarbituric acid reaction, based on the method of Eslami *et al.* (2016) with modified sample preparation. A total of 250 μL sperm was added to 2000 μL MDA solution (625 μL of 40% TCA, 100 μL of 1 N HCL, 50 μL of 1% Na-thiobarbiturate, and 975 μL of distilled water), which was heated at 100ºC for 30 min, and centrifuged at 4000 rpm for 10 min. The supernatant was separated and distilled water was added, reaching a volume of 3 mL. The absorbance was observed with a spectrophotometer (Shimadzu UV-1800, Japan) at a wavelength of 532 nm on a spectrophotometer and measured.

Intracellular Ca^{2+} Measurement

Measurement of intracellular Ca^{2+} concentration in sperm was carried out on fresh sperm and post-thawing sperm based on Aziz *et al.* (2021), with modifications to the preparation process. A total of 50 μL sperm was added to 150 µL phosphate buffered saline (PBS) and centrifuged at 6000 rpm for 2 min. Subsequently, 10 μL pellet was taken, followed by adding 10 μL Fluo-3 (Sigma-Aldrich), which was incubated at room temperature for 30 min in the dark. The sample was added with PBS to a total suspension volume of 150 μL and washed 3 times with a centrifuge at 6000 rpm for 2 min for each wash. Pellet was diluted with 30 μL PBS, dropped onto a cover slip, and covered with another cover slip. This process was followed by analyzing Ca^{2+} concentration using a Confocal Laser Scanning Microscope (Olympus FV1000, Japan).

Statistical analysis

The data obtained were tested for normality using the Shapiro-Wilk test. Normally distributed data were tested by ANOVA and when the p-value was significant or smaller than 0.05, the testing was continued with Duncan's multiple range test. For data that were not normally distributed, the Kruskal Wallis test was carried out and when the p-value was significant or smaller than *0.05*, the Dunn test was continued. All data were analyzed using IBM SPSS statistics 25 software.

Results

Sperm motility and kinematic parameters

The results of this study showed that treatment had no effect ($P > 0.05$) on total motility and static sperm percentage before freezing, but significantly affected progressive and slow motility ($P < 0.01$) (Table 2). The addition of sorbitol alone increased progressive motility (58.2%) and reduced slow motility (13.62%), but when combined with BHT it caused a decrease in progressive motility (40.06%) but was still better than the control. On the other hand, the incorporation of only BHT before freezing showed no difference from the control in all parameters. After freezing, a significant treatment effect ($P < 0.05$) was observed on total motility and static sperm percentage, while on progressive and slow motility were highly affected ($P < 0.01$) (Table 2). The addition of sorbitol in the diluent increased total (63.52–65.22%) and progressive motility (22.34–27.05%), followed by reduced slow motility and the percentage of static sperm post-thawing. Subsequently, the addition of BHT did not cause differences in all observed motility parameters except when combined with sorbitol.

Table 3 shows the results of sperm kinematic parameters observation before freezing, implying a highly significant treatment effect ($P < 0.01$) on DAP, DSL, DCL, VAP, VSL, VCL, LIN, ALH, BCF, and WOB. while STR has no substantial effect ($P > 0.05$). The addition of sorbitol alone increased DAP, DSL, DCL, VAP, VSL, VCL, LIN, ALH, and WOB, with decreasing BCF. The addition of BHT alone did not cause a difference from the control but was significantly different from the control when combined with sorbitol in DAP, DSL, DCL, ALH and BCF. The results of post-thawing sperm kinematic observations showed that diluent treatment had a very significant effect ($P < 0.01$) on DAP, DSL, DCL, VAP, VSL, VCL, ALH, BCF, STR, LIN and WOB. The addition of sorbitol alone or in combination with BHT increased DAP, DSL, DCL, VAP, VSL, VCL, STR, ALH and WOB, while BCF reduced significantly.

Table 4 shows that diluent treatment had no effect ($P > 0.05$) on sperm viability before freezing but significantly affected post-thawing sperm viability ($P < 0.05$). The addition of sorbitol in the diluent increased post-thawing sperm viability (67.69%), while BHT alone was unable to increase sperm viability unless combined with sorbitol. The combination of BHT and sorbitol did not cause a significant difference in viability compared to sorbitol alone (67.92%).

The results (Fig. 1) showed that the diluent treatment had a very significant effect ($P < 0.01$) on the post-thawing MDA concentration. The addition of sorbitol alone or in combination with BHT was able to reduce MDA concentration (0.7–0.91 μM), while BHT alone did not reduce MDA concentration.

Based on the results (Fig. 2), treatment had a very significant effect ($P < 0.01$) on sperm intracellular Ca^{2+} concentration. In fresh sperm, the Ca^{2+} concentration, namely 39.13 au, increased after freezing (73.02–84.48 au) without the presence of sorbitol in the diluent. The addition of sorbitol alone or in combination with BHT in the diluent did not cause a difference in Ca^{2+} concentration with fresh sperm (35.84–39.47 au).

Table 1: Composition of semen diluent used

Basic elements	Diluent treatment			
	Control	BHT	Sorbitol	BHT+Sorbitol
RLEY (%)	93	93	91	91
Penicillin (IU/mL)	1000	1000	1000	1000
Streptomycin (mg/mL)	1	1	1	1
DMSO (%)	7	7	7	7
BHT (m*M*)	-	3	-	3
Sorbitol (%)	-	-	2	2
pH	7.8	7.8	7.8	7.8
Osmolarity (mOsm/kg)	1586	1605	1615	1682

Table 2: Mean values of sperm motility parameters before freezing in Gaga chicken

	Motility Parameters (%)	Treatment			
		Control	BHT	Sorbitol	BHT + Sorbitol
Before freezing	Total motility	77.04 ± 4.55	79.67 ± 2.17	87.38 ± 2.80	82.71 ± 2.59
	Progressive motility	25.62 ± 1.96^{a}	26.04 ± 2.35^{a}	58.20 ± 5.63^{c}	40.06 ± 4.76^{b}
	Slow motility	31.26 ± 3.69^{b}	29.62 ± 4.95^{b}	13.62 ± 2.35^{a}	18.86 ± 3.54^{a}
	Static	22.96 ± 4.55	20.33 ± 2.17	12.62 ± 2.80	17.29 ± 2.59
Post thawing	Total motility	49.10 ± 4.23^{a}	51.37 ± 4.61^{ab}	63.52 ± 5.00^{b}	65.22 ± 4.60^{b}
	Progressive motility	1.64 ± 0.18^{a}	1.98 ± 0.38^{a}	22.34 ± 3.85^{b}	27.05 ± 4.36^{b}
	Slow motility	41.26 ± 4.28^{b}	38.64 ± 4.92^{b}	23.31 ± 2.87^{a}	21.28 ± 2.42^{a}
	Static	50.90 ± 4.23^{b}	48.63 ± 4.61^{ab}	36.48 ± 5.00^{a}	34.78 ± 4.60^{a}

Different superscripts on the same row indicate significant differences ($P < 0.05$)

Table 3: Mean values of sperm kinematic parameters before freezing in Gaga chicken

	Kinematic Parameters	Treatment			
		Control	BHT	Sorbitol	BHT + Sorbitol
Before freezing	DAP (μm)	16.16 ± 1.11^{a}	15.80 ± 0.82^{a}	26.57 ± 1.76^{c}	20.29 ± 1.24^{b}
	DSL (μm)	13.31 ± 1.13^{a}	12.75 ± 0.79^{a}	23.72 ± 1.83^{c}	17.01 ± 1.15^{b}
	DCL (μm)	28.06 ± 1.28^{a}	28.04 ± 0.89^{a}	38.32 ± 1.86^{c}	33.33 ± 1.61^{b}
	VAP (μm/s)	41.16 ± 2.86^{a}	40.86 ± 2.94^{a}	74.22 ± 7.71^{b}	53.05 ± 4.13^{a}
	VSL (μm/s)	34.11 ± 2.74^{a}	33.42 ± 2.73^{a}	66.75 ± 7.57^{b}	44.94 ± 3.86^{ab}
	VCL (μm/s)	70.61 ± 3.86^{a}	71.11 ± 3.75^{a}	106.72 ± 8.64^{b}	86.16 ± 5.32^{ab}
	STR (%)	81.13 ± 2.61	80.27 ± 2.79	87.10 ± 2.22	85.42 ± 2.55
	LIN (%)	44.07 ± 1.52^{a}	42.00 ± 1.95^{a}	57.38 ± 3.67^{b}	47.94 ± 2.28^{a}
	ALH (μm)	3.94 ± 0.22^{a}	4.27 ± 0.20^{ab}	5.27 ± 0.38^{c}	4.90 ± 0.25^{bc}
	BCF (Hz)	31.20 ± 0.84^{b}	30.84 ± 0.71^{b}	26.96 ± 1.13^{a}	27.76 ± 1.02^{a}
	WOB (%)	54.34 ± 1.08^{a}	53.41 ± 1.58^{a}	65.41 ± 2.94^{b}	57.59 ± 1.90^{a}
Post thawing	DAP (μm)	6.51 ± 0.24^{a}	6.94 ± 0.61^{a}	15.34 ± 1.07^{b}	17.59 ± 0.93^{b}
	DSL (μm)	4.25 ± 0.16^{a}	4.95 ± 0.69^{a}	11.82 ± 0.91^{b}	14.17 ± 1.14^{b}
	DCL (μm)	16.27 ± 0.64^{a}	16.04 ± 0.61^{a}	27.78 ± 1.63^{b}	29.26 ± 0.62^{b}
	VAP (μm/s)	15.56 ± 0.73^{a}	16.58 ± 1.43^{a}	41.22 ± 3.47^{b}	45.67 ± 2.84^{b}
	VSL (μm/s)	10.15 ± 0.45^{a}	11.91 ± 1.61^{a}	32.66 ± 3.13^{b}	37.30 ± 3.18^{b}
	VCL (μm/s)	38.19 ± 1.76^{a}	37.93 ± 1.58^{a}	73.36 ± 5.16^{b}	74.96 ± 2.59^{b}
	STR (%)	65.70 ± 2.15^{a}	69.77 ± 3.05^{a}	78.20 ± 2.52^{b}	79.58 ± 2.94^{b}
	LIN (%)	30.26 ± 1.37^{a}	34.72 ± 3.46^{ab}	39.89 ± 1.37^{bc}	44.43 ± 3.57^{c}
	ALH (μm)	2.71 ± 0.18^{a}	2.63 ± 0.15^{a}	4.53 ± 0.31^{b}	4.38 ± 0.23^{b}
	BCF (Hz)	35.84 ± 0.93^{b}	36.60 ± 1.00^{b}	31.70 ± 1.06^{a}	32.27 ± 1.07^{a}
	WOB (%)	41.82 ± 1.11^{a}	44.93 ± 2.79^{a}	52.27 ± 1.16^{b}	56.34 ± 2.67^{b}

Different superscripts on the same row indicate significant differences ($P < 0.05$)

Table 4: Mean values of sperm viability before freezing and post thawing in Gaga chicken

Parameter	Treatment			
	Control	BHT	Sorbitol	BHT + Sorbitol
Sperm viability before freezing (%)	95.45 ± 1.04	96.54 ± 0.89	97.41 ± 0.47	96.98 ± 0.71
Sperm viability post thawing (%)	52.75 ± 3.40^{a}	56.00 ± 3.85^{a}	67.69 ± 4.21^{b}	67.92 ± 4.06^{b}

Different superscripts on the same row indicate significant differences ($P < 0.05$)

However, the results obtained were lower when compared to the control and the addition of BHT alone. Fig. 3 shows an even distribution of intracellular Ca^{2+} in fresh sperm with the same pattern as post-thawing in the presence of sorbitol in the diluent.

Discussion

This study was carried out to determine the effect of adding sorbitol and BHT to the diluent on motility, kinematics, viability, lipid peroxidation, and intracellular Ca^{2+}

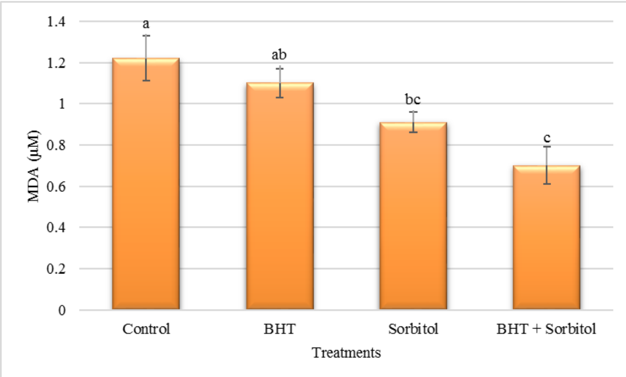

Fig. 1: MDA concentration in Gaga chicken semen post thawing

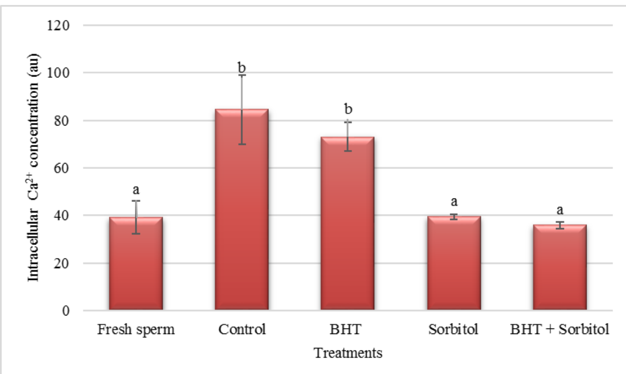

Fig. 2: Intracellular Ca^{2+} concentration in fresh and post-thawing Gaga chicken sperm (control, BHT, sorbitol and BHT+ sorbitol)

concentration in Gaga chicken sperm. This is the first study to analyze the effect of adding sorbitol in diluent on chicken sperm and the effect of 3 m*M* BHT on chicken sperm. The results showed that sorbitol had a positive effect on Gaga chicken sperm before freezing and post-thawing in almost all parameters observed. Meanwhile, adding BHT did not have a positive effect unless combined with sorbitol.

Before freezing, sorbitol increased the percentage of progressive motility, suppressed slow motility and influenced several sperm kinematic parameters. This phenomenon may be attributed to sorbitol serving as a source of energy for sperm, including glucose and fructose. Storey (2008) reported that sorbitol can act as an alternative energy substrate for glycolysis and oxidative phosphorylation in mammalian sperm. Visconti (2012) also indicated its potential to maintain a high percentage of motile sperm in the glycolytic pathway, supporting increased tyrosine phosphorylation. In this study, the high motility before freezing with sorbitol treatment was caused by conversion into fructose, a source of energy for sperm movement. Sorbitol can be converted into fructose by the enzyme sorbitol dehydrogenase, allowing penetration into sperm plasma membrane (Frenette *et al.* 2006; Cao *et al.* 2009). Generally, transporters on the plasma membrane move derived fructose into sperm, which are used as an energy source for glycolysis and oxidative phosphorylation (Cao *et al.* 2009). In chicken sperm, glycolysis, and oxidative phosphorylation produce adenosine triphosphate (ATP) as an energy source for sperm motility (Setiawan *et al.* 2020). According to Stanishevskaya *et al.* (2021b), fructose is a sugar that easily penetrates chicken sperm cell membranes, creating additional energy reserves to maintain sperm motility. In this study, total motility with the addition of sorbitol and the combination of sorbitol with BHT before freezing were 82.71–87.38%. These results were higher compared to 76.6–77 and 74.14–80.62% obtained in the Ross breed, sperm treated with the addition of mitoquinone (Nazari *et al.* 2022) and Hisex white chicken with fetal bovine serum (FBS) in the diluent (Blank *et al.* 2020), respectively.

The addition of sorbitol before freezing was able to increase the velocity of chicken sperm as seen in VCL, VSL, and VAP. This improvement was attributed to metabolized sorbitol producing more ATP, crucial for sperm velocity, but requiring further investigation. Setiawan *et al.* (2021) reported that glucose supplementation in the medium increased ATP in the cytoplasm of chicken sperm cells. This phenomenon was in line with previous studies on rodent sperm, where a positive relationship was observed between ATP concentration and sperm velocity. Furthermore, it was observed that extra stored ATP can enhance swimming sperm speed (Tourmente *et al.* 2013), but the effect varied across species. The addition of sorbitol alone also increased LIN, ALH and WOB in chicken sperm. High LIN and STR values showed progressive swimming sperm, while high ALH values showed hyperactive sperm (Ratnawati and Luthfi 2020). In this study, VCL, VAP and VSL of sperm before freezing were 70.61–106.72 μm/s, 41.16–74.22 μm/s, and 34.11–66.75 μm/s, respectively. However, these values were lower compared to Blank *et al.* (2020), who reported 133.7 μm/s, 97 μm/s and 77.7 μm/s, respectively, with FBS treatment. ALH and LIN were close to the values reported by Nazari *et al*. (2022) at 5.56 μm and 26.62%, respectively. Moreover, BCF before freezing was also close to the report by Blank *et al.* (2020), namely 25.61–27.1 Hz.

At the post-thawing stage, sorbitol increased total motility, progressive motility, viability, and almost all kinematic parameters. This occurred because sorbitol, as a type of sugar alcohol, functions as an energy source, and cryoprotectant. According to Zhu *et al.* (2023), sugar alcohols have great potential as cryoprotectants due to the high content of hydroxyl groups compared to saccharides, facilitating the inhibition of large crystal formation. Takahashi and Hatta (2001) found that sorbitol interacted with phosphatidylcholine, the main component of biomembranes, reducing the interface area between the lipid and water phases.

Post-thawing total motility in sorbitol treatment and its combination with BHT in this study was higher than

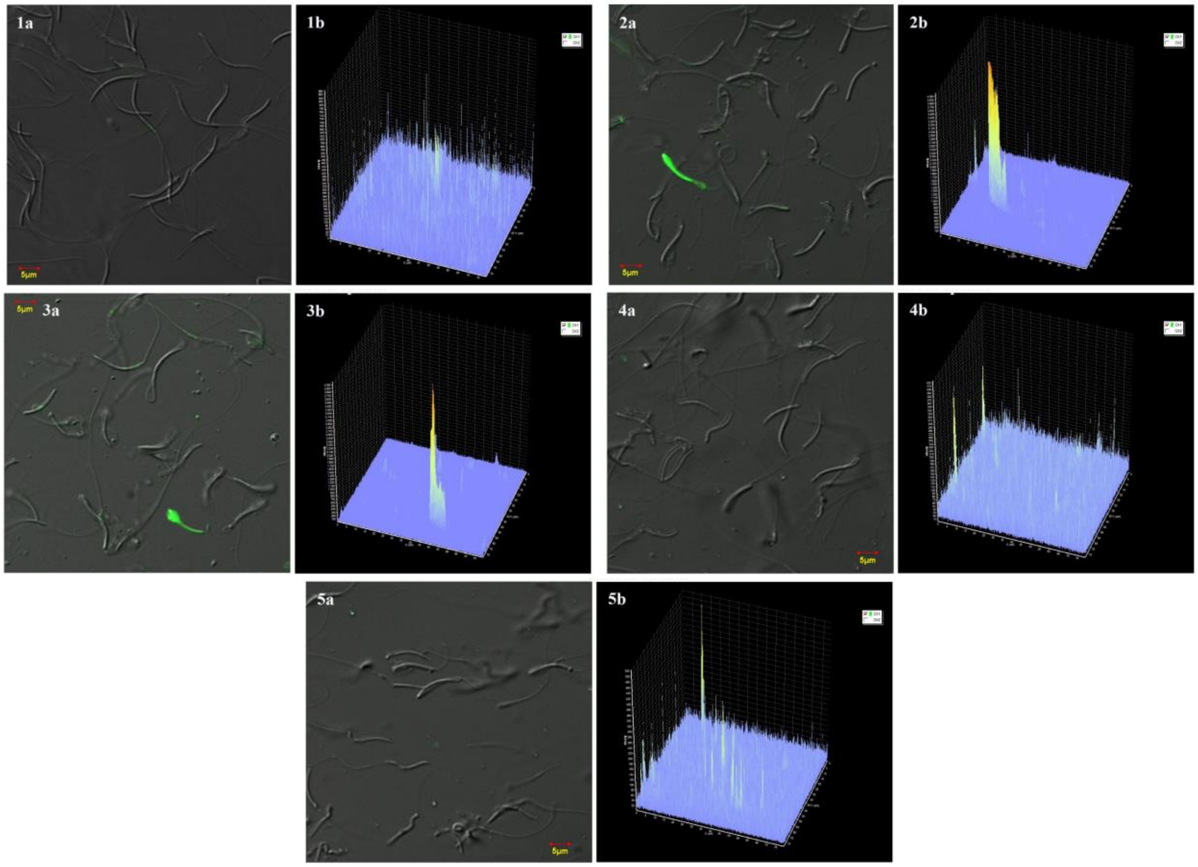

Fig. 3: CLSM analysis results: **1**: Fresh sperm, **2**: control post thawing sperm, **3**: post thawing sperm with BHT treatment, **4**: post thawing sperm with sorbitol treatment, **5**: post thawing sperm with a combination of sorbitol and BHT. **a**: Super infusion image of sperm, **b**. Intracellular Ca^{2+} intensity in sperm

previous studies, namely 57.84 and 48–50% in Gaga chicken sperm with sucrose treatment (Khaeruddin *et al.* 2022) and Rhode Island Red breed with trehalose treatment (Stanishevskaya *et al.* 2021b), respectively. Similarly, the progressive motility of post-thawing sperm treated with sorbitol and its combination with BHT was higher than previous studies, namely 14.5% in PD 6 Line chicken sperm treated with 1 mM BHT (Kumar *et al.* 2019) and 9.7% in Greenleg Partridge breed with melatonin treatment (Mehaisen *et al.* 2020). Post-thawing sperm viability was higher than previous reports, namely 31.91% (sucrose treatment) and 42.83% (hyaluronic acid treatment) in Gaga chicken sperm (Khaeruddin *et al.* 2022), 58% in Ross broiler sperm with hyaluronic acid treatment (Lotfi *et al.* 2017), as well as 19.45% (1 mM BHT treatment) and 31.6% (vitamin E treatment) in PD 6 Line chicken sperm (Kumar *et al.* 2019).

The addition of sorbitol and its combination with BHT reduced lipid peroxidation in sperm which was shown by low post-thawing MDA concentrations. Generally, MDA is formed from the decomposition of monocyclic peroxides, resulting from lipid peroxyl radicals passing through cyclization (Yekti *et al.* 2018). Mussa *et al.* (2020) stated that lipid peroxidation, as shown by high concentrations of MDA, caused a decrease in chicken sperm motility. The results showed that sorbitol also had antioxidant activity for sperm. According to Kang *et al.* (2007), sugar alcohols have antioxidant activity by preventing cells from oxidative stress through scavenging oxyradicals. This antioxidant capacity depends on the number of aliphatic hydroxyl groups (Kang *et al.* 2007), with sorbitol consisting of six groups in its structural formula (Li *et al.* 2021; Silva *et al.* 2021). According to Hincha and Hageman (2004), sugar alcohol can stabilize the sperm plasma membrane when exposed to oxidative stress.

Intracellular Ca^{2+} concentrations have been widely studied in mammalian sperm, with limited attention given to fowl sperm. This study is the first to examine intracellular Ca^{2+} concentrations in chicken frozen sperm using confocal microscopy and the Fluo-3 fluorescent indicator. Previous investigations only tested Ca^{2+} content using a spectrofluorimeter with the fluorescent indicator Fura-2, where the intracellular Ca^{2+} concentration greatly influenced sperm motility and respiration (Ashizawa *et al.* 1992). A recent report also showed that Ca^{2+} homeostasis is essential for maintaining chicken sperm motility *in vitro* (Froman

2016) and Ca^{2+} channels in the membranes regulate motility and acrosome reactions (Nguyen *et al.* 2016).

In all eukaryotic cells, Ca^{2+} signal transduction regulates several cellular functions, including transcription, enzyme activity, protein phosphorylation and cell death (Carafoli 2002). An increase in intracellular calcium also occurs through the release of Ca^{2+} from intracellular stores or the influx across the plasma membrane (Nguyen *et al.* 2016). In this study, freezing caused an increase in intracellular Ca^{2+} in chicken sperm, except with the addition of sorbitol in the diluent. The increase in membrane permeability due to cooling allowed Ca^{2+} to penetrate cells (Robertson and Watson 1987), while depolarization induced the entry process (Kim *et al.* 2018). A similar increase in intracellular Ca^{2+} after freezing has also been reported in bovine (Treulen *et al.* 2018) and stallion sperm (Yeste *et al.* 2015).

The increase in intracellular Ca^{2+} is inversely proportional to motility. This is shown by lower sperm motility post-thawing in the control treatment with a higher Ca^{2+} concentration than in the sorbitol treatment. Meanwhile, an increase in intracellular Ca^{2+} directly or due to increased ROS production causes a decrease in sperm motility (Keshtgar *et al.* 2016). The decrease in motility is caused by increasing Ca^{2+} concentrations, and leads to a reduction in protein phosphorylation, thereby preventing substrate-kinase interactions (Tash and Means 1983).

Schuh *et al.* (2004) stated that Ca-ATPase was required for the regulation of sperm function and intracellular Ca^{2+} levels. Generally, cooling causes membrane damage due to hydrolysis and Ca^{2+}-ATPase function (Sieme *et al.* 2015; Jin and Yang 2017). In this study, although the use of sorbitol maintained Ca^{2+} concentrations after freezing, the mechanism still required further investigation. This phenomenon may be attributed to the ability of sorbitol to maintain Ca^{2+}-ATPase activity in sperm membranes. According to Yoo (2014), sorbitol is capable of maintaining Ca^{2+}-ATPase activity in frozen surimi. The results show the effectiveness of sorbitol in maintaining chicken sperm quality, offering potential application as a basis for cryopreservation programs in the poultry industry.

Conclusion

In conclusion, this study showed that the addition of sorbitol in the diluent produced the best sperm quality and maintained intracellular calcium concentration during frezee-thaw process. The results also showed that sorbitol and its combination with BHT treatment prevented lipid peroxidation in Gaga chicken frozen sperm, while BHT alone had no significant effect.

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Author Contributions

KK: Semen collection, making diluent, freezing semen, evaluating viability and MDA, data analysis, and writing publication manuscripts, GC: methodology and supervision, MY: methodology and supervision, WS: analyzing sperm movement with computer assisted sperm analysis, CC: carried out intracellular calcium analysis using a confocal laser scanning microscope and data interpretation. SW: methodology and supervision.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

This study has been approved by the University of Brawijaya Research Ethics Committee with Approval No: 020-KEP-UB-2023.

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