2024, Scienceline Publication
World's Veterinary Journal
World Vet J, 14(3): 338-348.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2024.wvj40
PII: S232245682400040-14

Effects of Butylated Hydroxytoluene and Sorbitol as Diluent Components on Structural and Surface Ultrastructural Changes of Gaga Chicken Sperm During Cryopreservation

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ORIGINAL ARTICLE
Received: June 29, 2024
Revised: July 20, 2024
Accepted: August 20, 2024
Published: September 25, 2024

ABSTRACT

The Gaga chicken is an indigenous Indonesian breed that is important to preserve using semen cryopreservation technology. The study was conducted to determine the effect of adding sorbitol and butylated hydroxytoluene (BHT) in the diluent on the structural and surface ultrastructure of cryopreserved Gaga chicken sperm during cryopreservation /frozen storage. The study aimed to assess how adding sorbitol and butylated hydroxytoluene (BHT) to the diluent affects the structure and surface ultrastructure of cryopreserved Gaga chicken sperm. A completely randomized design was employed with four treatments and 10 replications including egg yolk-lactate ringer diluent (EYLR) as the control group, EYLR diluent with 3 mM BHT, EYLR diluent with 2% sorbitol, and EYLR diluent with both 3 mM BHT and 2% sorbitol. Semen was collected using a massage technique from 4 male chickens aged approximately 10 months, pooled semen was diluted, packaged in 0.25 mL straws, equilibrated for 2 hours at 5 °C, pre-freeze for 10 minutes, frozen for 24 hours, and thawed for 30 seconds at 37 °C. The parameters evaluated were sperm plasma membrane integrity, acrosome integrity, DNA damage, mitochondrial functionality, and surface ultrastructure. The results showed that the treatment had a significant effect on plasma membrane integrity and post-thawing mitochondrial functionality compared to the control, but no effect was observed on acrosome integrity or DNA damage. The results showed that the combination treatment of BHT with sorbitol had a significant effect on plasma membrane integrity and post-thawing mitochondrial function, but did not affect acrosome integrity or DNA damage when compared to the control group. Ultrastructural observations indicated that cryopreservation caused damage to the head, middle, and tail of the sperm in the control groups. However, these changes were prevented by the diluent containing a combination of BHT and sorbitol. The addition of both components (BHT 3 mM + sorbitol 2%) effectively maintained plasma membrane integrity, mitochondrial functionality, and surface ultrastructure of Gaga chicken sperm during cryopreservation.

Keywords: Butylated hydroxytoluene, Chicken sperm, Cryopreservation, Sorbito, Structure, Sperm ultrastructure

INTRODUCTION

Gaga chicken, an original Indonesian breed, is renowned for a relatively long sound resembling human laughter, leading to the preference by hobbyists for competitions. In this context, longer-duration vocalizations often fetch higher prices (Khaeruddin et al., 2024a). Gaga chicken mating system in society occurs uncontrollably, allowing the decline in purity due to natural mating with other local chicken species (Bugiwati and Ashari, 2013). The Decree of the Minister of Agriculture number 2920/Kpts/OT.140/6/2011, Indonesia has mandated the protection and preservation of Gaga chicken as a rich livestock genetic resource (Khaeruddin et al., 2022).

Cryopreservation is a method to preserve germplasm in various fields including endangered animal conservation, through freezing spermatozoa (Sharafi et al., 2022). Storing chicken sperm by freezing (-196 °C) for 18 years does not reduce the ability to fertilize egg cells (Thélie et al., 2019). However, several reports indicated low sperm quality in chickens after freeze-thawing (Kumar et al., 2019; Masoudi et al., 2021; Ratchamak et al., 2023).

Cell damage during the cryopreservation process is caused by the formation of ice crystals as well as chemical and physical processes, including the denaturation of proteins and lipids in membranes, which trigger the formation of reactive oxygen species (ROS, Pini et al., 2018). Cryopreservation causes low plasma membrane and acrosome integrity as well as post-thawing mitochondrial activity in chicken sperm (Partyka et al., 2012). Ultrastructural analysis by Zong et al. (2023) found that cryopreservation also caused structural damage, particularly in the mitochondria, midpiece, and perforatorium.

To cite this paper: Khaeruddin, Ciptadi G, Yusuf M, Suyadi, Natsir MH, Hermadi HA, and Wahjuningsih S (2024). Effects of Butylated Hydroxytoluene and Sorbitol as Diluent Components on Structural and Surface Ultrastructural Changes of Gaga Chicken Sperm During Cryopreservation. *World Vet. J.,* 14(3): 338-348. DOI: https://dx.doi.org/10.54203/scil.2024.wvj40

Damage to spermatozoa during the cryopreservation process can be prevented by adding special ingredients to the semen diluent. Cryoprotectants in retailers are needed to protect spermatozoa cells both extracellularly and intracellularly. Examples of extracellular cryoprotectants are dimethyl sulfoxide and glycerol, while extracellular cryoprotectants include several types of sugar, such as trehalose (Murray and Gibson, 2022), sucrose, lactose, glucose or fructose (Gómez-Fernández et al., 2012). The addition of sugar has proven effective as a cryoprotectant agent on chicken spermatozoa (Thananurak et al., 2019; Stanishevskaya et al., 2021). Apart from being a cryoprotectant, sugar also acts as an energy source (Zhang et al., 2023; Gholami et al., 2023). Previous reports showed that sugar alcohols such as sorbitol were more effective in maintaining mammalian sperm quality during cryoprotection (Pojprasath et al., 2011; Wu et al., 2016), but this finding has not been reported for avian sperm.

Poultry sperm cell membranes, rich in polyunsaturated fatty acids (PUFA, Mussa et al., 2021), are susceptible to damage from ROS (Collodel et al., 2022). Additional antioxidants can reduce the impact of sperm damage due to ROS and cold shock, improving post-thawing semen quality (Amidi et al., 2016). Butylated hydroxytoluene (BHT), an antioxidant, effectively reduces ROS levels (Zhao et al., 2018), as evidenced by the ability to maintain the quality of mammalian sperm during the cryopreservation process (Merino et al., 2015; Seifi-Jamadi et al., 2016; Jara et al. 2019; Sun et al., 2020). Previous studies reported the effectiveness in maintaining the quality of Gaga chicken sperm during liquid storage (Khaeruddin et al., 2024a). Despite the widespread usage, the effect of sorbitol and combination with BHT on the quality of chicken sperm during the cryopreservation process has not been reported. Therefore, the current study aimed to assess how adding sorbitol and butylated hydroxytoluene (BHT) to the diluent affects the structure and ultrastructure of Gaga chicken sperm during freeze-thawing.

MATERIALS AND METHODS

Ethical approval

The University of Brawijaya Research Ethics Committee, Indonesia, granted Approval No: 020-KEP-UB-2023 for the procedures and animals used in this study.

Diluent preparation

The primary diluent utilized was egg yolk-Ringer's lactate (EYRL), consisting of 90% Ringer's lactate and 10% chicken egg yolk, which was homogenized and centrifuged at 3000 rpm for 15 minutes. The Ringer's lactate solution (PT. Widatra Bakti, Indonesia) contained 3 g sodium chloride, 1.55 g sodium lactate, 0.1 g calcium chloride, and 0.155 g potassium chloride dissolved in 500 ml of sterile water. After centrifugation, the supernatant was collected, and 1000 IU penicillin (PT Meiji, Indonesia), 1 mg/ml streptomycin (PT Meiji, Indonesia), and 7% dimethyl sulfoxide (Merck KGaA, Germany) were added. Subsequently, the diluent was divided into 4 tubes with 3 mM BHT (Sigma, US) and 2% sorbitol (Merck KGaA, Germany) added. The treatment composition was EYRL (control, osmolality 1586 mOsm/kg), EYRL + BHT (osmolality 1605 mOsm/kg), EYRL + sorbitol (osmolality 1615 mOsm/kg), and EYRL + BHT + sorbitol (osmolality 1682 mOsm/kg).

Chicken maintenance and semen collection

Four Gaga chickens aged 12 months obtained from breeders in Malang Regency, East Java, Indonesia were used as a source of semen for cryopreservation. The chickens were kept in individual cages measuring 55 x 60 x 60 cm3 and were given commercial feed (PT New Hope, East Java, Indonesia, 17% crude protein, 2800-2900 kcal/kg energy metabolism, 3% crude fat, and 14% ash) at 100 gr/day alongside drinking water *ad libitum*. Semen was collected using tuberculin spoid by massaging each individual (Kucera and Heidinger, 2018). The Semen collected from each individual was evaluated macroscopically and microscopically, and semen that met the requirements (motility > 70%, viability > 90%, and abnormalities < 20%) were used for the next stage. The semen collected from each individual ranged from 0.1 to 0.2 ml, which was mixed (pooled) was processed further to the dilution and cryopreservation stages.

Cryopreservation stages

Semen was divided into 4 microtubes and diluted based on treatment followed by packaging with a minimum concentration of 200 million per straw (IMV, France). The straw was equilibrated at a temperature of 5 °C for 2 hours (Wahjuningsih et al., 2024), then pre-freezing was carried out in a styrofoam box by placing the straw 3 cm above the surface of liquid nitrogen (Madeddu et al., 2016) for 10 minutes (Mosca et al., 2016). The straw was equilibrated at 5°C for 2 hours (Wahjuningsih et al., 2024). Following this, pre-freezing was performed by placing the straw 3 cm above the surface of liquid nitrogen in a styrofoam box (Madeddu et al., 2016) for 10 minutes (Mosca et al., 2016). Subsequently, the straw was stored in a tank filled with liquid nitrogen (-196 °C) for 24 hours and thawed in a water bath for 30 seconds in water at 37°C (Shah et al., 2016; Wahjuningsih et al., 2024).

Semen evaluation

Plasma membrane integrity

Plasma membrane integrity was assessed using a hypoosmotic swelling test (HOST) solution, which contained 0.9 g of fructose and 0.49 g of sodium citrate in 100 µl of distilled water. Approximately 10 µl of liquid semen was mixed with 100 µl of HOST solution and incubated at 37°C for 30 minutes (Mehdipour et al., 2016; Najafi et al., 2019). The solution was then fixed onto a glass slide using eosin-nigrosin solution, dried, and observed under a light microscope (Olympus CX23, Japan) at 400x magnification (Figure 1). Sperm with intact plasma membranes were identified according to the criteria outlined by Santiago-Moreno et al. (2009), including bent tail, knotted tail tip, bent midpiece, as well as shortened and thickened tail.

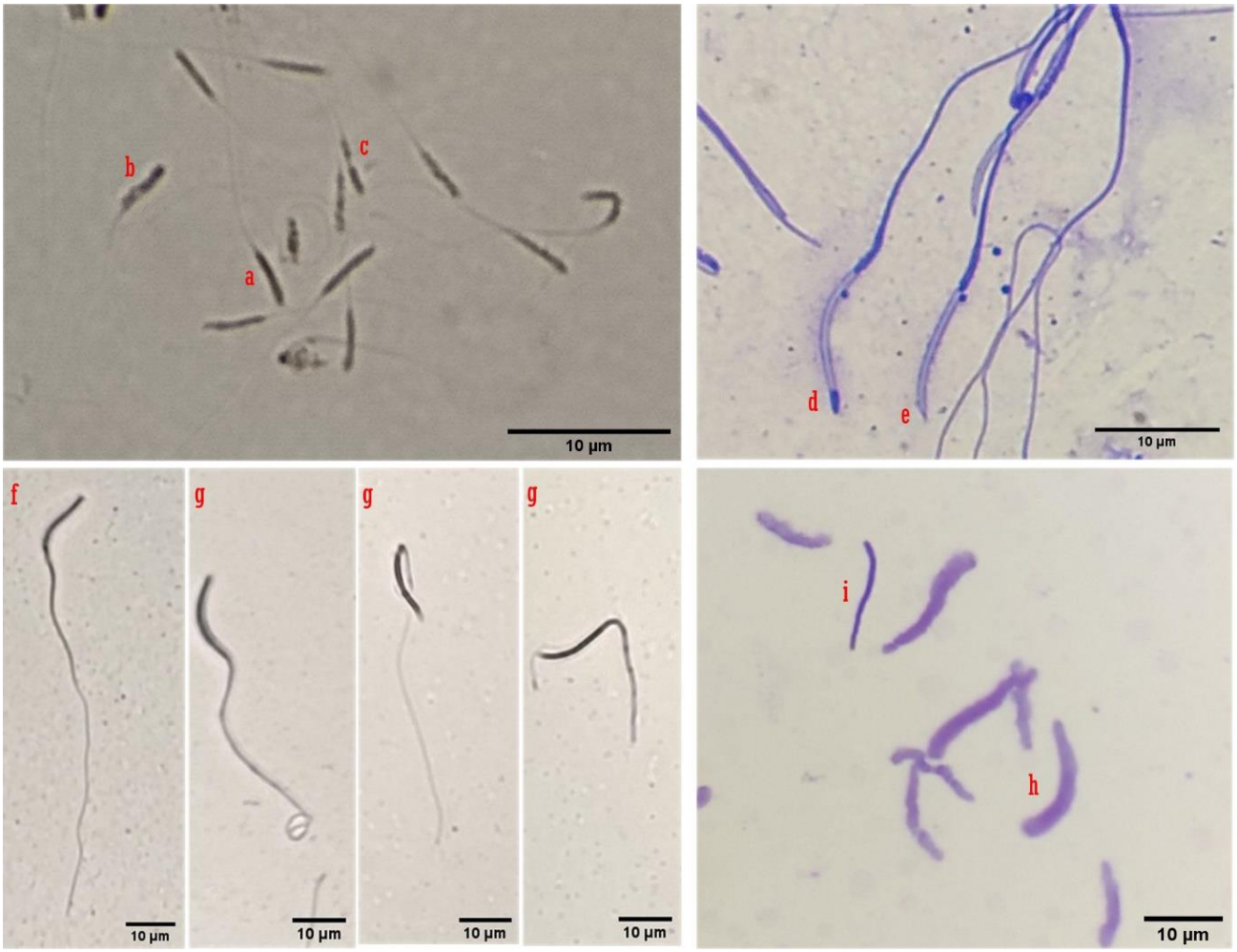


Figure 1. Gaga chicken sperm structure using a light microscope (with 1000x magnification). Mitochondrial functionality (**a**: DAB I, **b**: DAB II, **c**: DAB III), acrosome integrity (**d**: Intact, **e**: Damaged), plasma membrane integrity (**f**: intact membrane, **g**: damaged membrane) and DNA damage (**h**: Intact, **i**: Damaged)

Acrosome integrity

A drop of semen was placed on a glass slide, mixed with 5% formalin, and then thinly smeared on the slide. The samples were re-fixed in a 5% formalin solution for 30 minutes at 37°C, then rinsed and air-dried (Khaeruddin et al., 2024a). Following this, the samples were stained using Coomassie Brilliant Blue solution [0.25% Coomassie Brilliant Blue R 250 (BBI Life Sciences, Canada) in a solution of 25% methanol and 10% glacial acetic acid] for 5 minutes, followed by rinsing and air-drying. The observation was conducted under a light microscope at 1000x magnification (dropped with immersion oil) (Figure 1), with intact acrosomes appearing blue and thick while incomplete acrosomes were less colored or thin (Silyukova et al., 2022).

Mitochondrial functionality

Mitochondrial functionality was observed using 3,3'-diaminobenzidine (DAB) dye (Sigma-Aldrich, US). Semen was diluted in a DAB solution (1 mg/mL phosphate-buffered saline), covered with aluminum foil, and incubated for 1

hour at 37°C. Approximately 10 µl of the solution was then smeared on a glass slide and air-dried. Subsequently, observation was conducted using a light microscope with 1000x magnification (dropped with immersion oil) on 200 sperm cells (Figure 1). Mitochondrial activity was divided into 4 categories, namely DAB I (100% active), DAB II (> 50% active), DAB III (< 50% active), and DAB IV (inactive) (Rui et al., 2017).

DNA damage

DNA damage was observed using toluidine blue O dye (Merck KGaA, Germany), in this process, semen was spread thinly on a glass object and air-dried. The preparation was fixed for 30 minutes in a solution of 96% ethanol-acetone (1:1) at 4 °C and air-dried. Subsequently, hydrolysis was carried out for 5 minutes in 0.1 N HCl solution at a temperature of 4 °C, followed by rinsing three times with an interval of 2 minutes and air drying. The preparation was dripped with toluidine blue O and left at room temperature for 20 minutes, then rinsed and air dried. Observations were carried out under a light microscope at 400x magnification (Figure 1). Sperm heads with damaged DNA were marked in dark blue while intact DNA was colored bright blue (Rui et al., 2017; Khaeruddin et al., 2024a).

Sperm ultrastructural analysis

Sperm ultrastructural analysis was performed using field-emission scanning electron microscopy (FE-SEM) (FEI Quanta 600, US). The sample preparation involved washing the semen with physiological NaCl, and then fixing it with 2.5% glutaraldehyde for 3-4 hours. This was followed by washing with pH 8 phosphate buffer saline (PBS) three times for 5 minutes each, dehydrating with graded alcohol, and then attaching to a stub. Subsequently, the stub was attached and coated with gold. The sample was analyzed by FE-SEM (FEI Quanta 600, US) at 20,000x magnification with an acceleration voltage of 10 kV.

Statistical analysis

This study used a completely randomized design with 4 treatments and 10 replications. Sperm quality data was analyzed for variance (ANOVA), and Duncan's multiple range test was performed when a significant effect ($p < 0.05$) was found. Ultrastructural data was analyzed descriptively and all analyses were conducted using IBM SPSS Statistics 25 software.

RESULTS

Plasma membrane integrity

The results indicated that adding sorbitol and BHT to EYLR did not significantly impact ($p > 0.05$) the plasma membrane integrity of Gaga chicken sperm before freezing, but a significant effect was observed after thawing ($p < 0.05$, Graph 1). The addition of BHT and sorbitol in the diluent effectively maintained post-thawing plasma membrane integrity (65.47-70.02%) compared to control groups (58.43%). Before freezing, the plasma membrane integrity was in the range of 97.22-98.35%, but a decrease was found post-thawing with a range of 58.43-70.02%.

Acrosome integrity

The addition of BHT and sorbitol in the diluent did not affect the acrosome integrity of Gaga chicken sperm before freezing and post-thawing ($p > 0.05$, Table 1). The range of acrosome integrity before freezing was 97.23-97.9% which decreased to 40.95-42.57% post-thawing.

DNA damage

The results showed that the addition of BHT and sorbitol in EYLR diluent did not affect sperm DNA damage before freezing and post-thawing ($p > 0.05$, Table 2). The range of DNA damage before freezing was relatively low, namely 1.49-1.86, which increased slightly post-thawing to 2.59-3.14%.

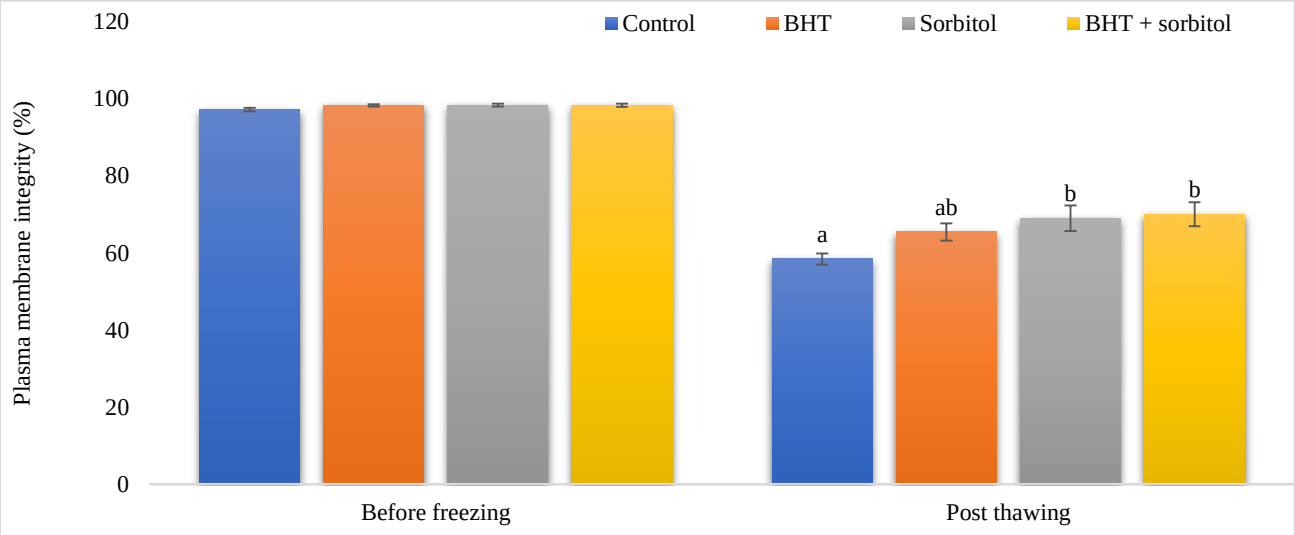
Mitochondria functionality

The addition of BHT and sorbitol in the diluent did not affect the mitochondrial functionality of sperm ($p > 0.05$) before freezing but had a significant effect on post-thawing ($p < 0.05$, DAB I and DAB II, Graph 2). The range of DAB I (100% active mitochondria) before freezing decreased post-thawing, where the addition of sorbitol and combination with BHT in the diluent produced a higher percentage, namely 58.9-63.59%.

Sperm surface ultrastructure

The ultrastructural analysis results of spermatozoa using FE-SEM (Figure 2 a-i) showed that chicken sperm before freezing (fresh semen) had an intact head and acrosome (Figure 2a), as well as sperm post thawing with the addition of BHT + sorbitol (Figure 2b), while post-thawing sperm without BHT and sorbitol experienced damage to the head (Figure 2c). The midpiece of the sperm before freezing looked intact (Figure 2d), as did the post thawing sperm

with the addition of BHT + sorbitol (Figure 2e), while the midpiece of the sperm post thawing without BHT and sorbitol was damaged (Figure 2f). The sperm tail before freezing looked intact (Figure 2g), as did the sperm post thawing with the addition of BHT + sorbitol (Figure 2h), while the sperm tail post thawing without BHT and sorbitol tended to experience damage (Figure 2i).



Graph 1. Plasma membrane integrity percentage of Gaga chicken sperm during cryopreservation with the addition of butylated hydroxytoluene (BHT) and sorbitol in the diluent

Table 1. Acrosome integrity percentage of Gaga chicken sperm before freezing and post-thawing with the addition of butylated hydroxytoluene (BHT) and sorbitol to the diluent

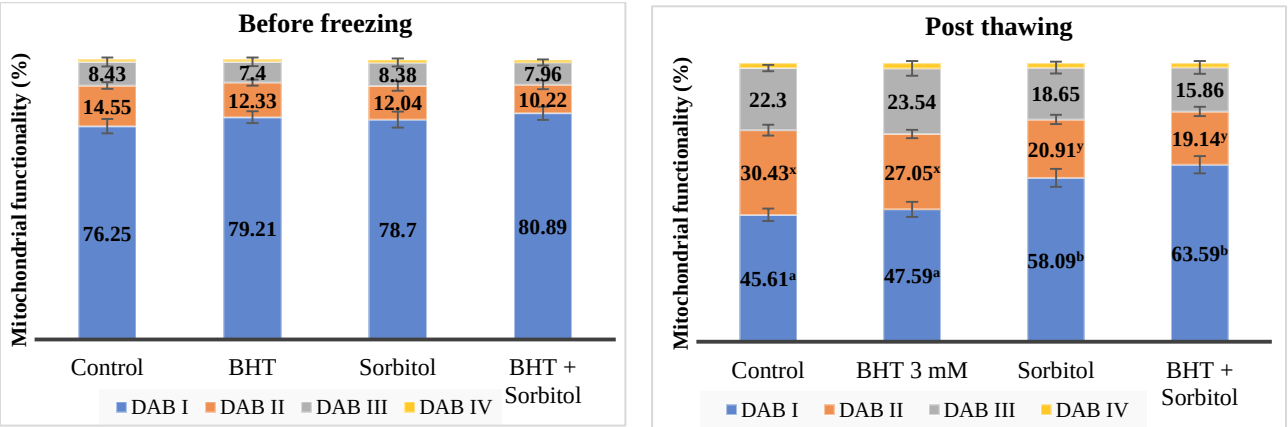
Treatments	Before freezing	Post thawing
Control	97.23 ± 0.69	40.95 ± 2.68
BHT	97.53 ± 0.50	42.22 ± 3.29
Sorbitol	97.78 ± 0.48	42.57 ± 3.33
BHT + Sorbitol	97.90 ± 0.52	42.55 ± 2.54

Control: EYLR diluent, BHT: EYLR diluent + 3 mM BHT, sorbitol: EYLR diluent + 2% sorbitol, and BHT + sorbitol: EYLR diluent + 3 mM BHT + 2% sorbitol.

Table 2. DNA damage percentage of Gaga chicken sperm before freezing and post-thawing with the addition of butylated hydroxytoluene (BHT) and sorbitol to the diluent

Treatments	Before freezing	Post thawing
Control	1.86 ± 0.20	3.14 ± 0.38
BHT	1.49 ± 0.11	3.01 ± 0.39
Sorbitol	1.74 ± 0.22	2.67 ± 0.35
BHT + Sorbitol	1.61 ± 0.23	2.59 ± 0.36

Control: EYLR diluent, BHT: EYLR diluent + 3 mM BHT, sorbitol: EYLR diluent + 2% sorbitol, and BHT + sorbitol: EYLR diluent + 3 mM BHT + 2% sorbitol.



Graph 2. Mitochondrial functionality percentage of Gaga chicken sperm during cryopreservation with the addition of butylated hydroxytoluene (BHT) and sorbitol in the diluent. **DAB I**: 100% mitochondria active, **DAB II**: > 50% mitochondria active, **DAB III**: < 50% mitochondria active, and **DAB IV**: Mitochondria inactive

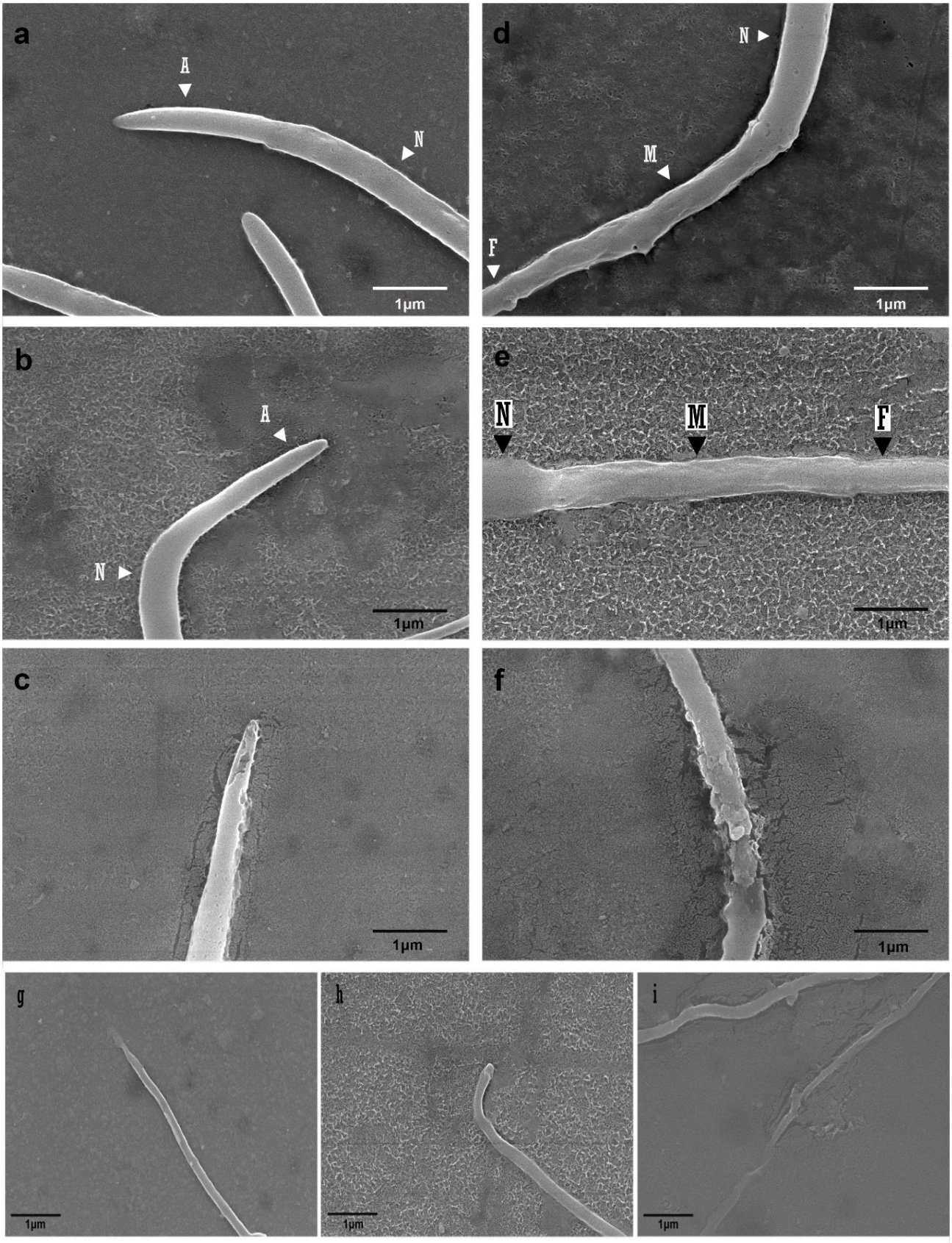


Figure 2. Surface ultrastructural observations of the Gaga sperm surface using the FE-SEM instrument with 20000x magnification. **a**: Sperm heads (fresh semen), **b**: Post-thawing sperm heads (butylated hydroxytoluene + sorbitol), **c**: Post-thawing sperm heads (control), **d**: Sperm midpiece (fresh semen), **e**: Sperm midpiece post thawing (butylated hydroxytoluene + sorbitol), **f:** Sperm midpiece post thawing (control), **g**: Sperm tail (fresh semen), **h**: Post thawing sperm tail (butylated hydroxytoluene + sorbitol), **i**: Post thawing sperm tail (control)

DISCUSSION

Analyzing the structure and ultrastructure of sperm after freezing is crucial as intact sperm are vital for successful fertilization in artificial insemination. This study was one of the first to examine the effects of sorbitol and BHT combination as a diluent on the structure and surface ultrastructure of Gaga chicken sperm in the cryopreservation process. Based on the results, the addition of sorbitol and combination with BHT effectively maintained the integrity of the plasma membrane post-thawing. Sorbitol acts as an antioxidant by donating hydrogen to free radicals, thereby preventing lipid peroxidation in the membrane.

According to Peshev et al. (2013), sugar can act as a hydroxyl (OH) scavenger through the transfer of hydrogen atoms. Sugar alcohol acts as an antioxidant by scavenging oxy-radicals thereby preventing oxidative stress in cells (Kang et al., 2007). More specifically, sorbitol functions by scavenging OHl radicals (Faraji and Linsay, 2004) and superoxide radicals up to 28% (Truffin et al., 2021). As cryoprotectants, sugar alcohols protect proteins from aggregation by inhibiting denaturation caused by ice crystal growth and preventing intermolecular hydrophobic interactions as cryoprotectants, sugar alcohols safeguard proteins from aggregation by preventing denaturation due to ice crystal formation and blocking intermolecular hydrophobic interactions (Zhu et al., 2023). Santivarangkna et al. (2010) suggest that sorbitol protects cells by suppressing the membrane phase transition temperature through interactions with phosphate groups. Takahashi and Hatta (2001) also stated that the compound reduced the interface area between the lipid and water phases. According to Hincha and Hageman (2004), sorbitol showed better protection against membrane leakage than disaccharides. It was proven more effective than other types of sugar in maintaining the integrity of sperm membranes after freezing (Wu et al., 2016). Sorbitol may play an important role in bacterial cell protection, by stabilizing the membrane bilayer or fluidity, and cell recovery from stress (Sootsuwan et al., 2013). Meanwhile, BHT, a phenolic compound is a synthetic antioxidant that eliminates or deactivates free radicals formed during initiation or propagation reactions, inhibiting chain reactions (Fasihnia et al., 2020).

The freezing process in this study caused a decrease in sperm membrane integrity post-thawing. The formation of ice crystals is one of the main sources of physical damage to sperm membranes (Upadhyay et al., 2021; Lee et al., 2023). Another cause of membrane damage in the cryopreservation process is excessive ROS production which triggers changes in membrane proteins, lipids, and carbohydrates due to reduced disulfide bonds between proteins, peroxidation of membrane phospholipids, and modification of the sperm glycocalyx. These changes cause sperm to become brittle and lose their semipermeable properties (Peris-Frau et al., 2020). The average post-thawing plasma membrane integrity value was higher than previous studies, which used Beltsville Poultry Semen Extender (BPSE) diluent added with lycopene-loaded nanoliposomes (42.46-62.71%, Najafi et al., 2018), and Lake diluent added with MitoQ (55-64%, Alipour-Jenaghard et al., 2023). However, the result was almost the same as Stanishevskaya et al. (2021) which obtained a value of 67.2-69.3% using Leningrad Cryoprotective Medium diluent added with disaccharides.

Based on the results, the addition of sorbitol and BHT to the diluent had no significant effect on acrosome integrity and DNA damage. Freezing caused a decrease in acrosome integrity and slightly increased DNA damage. Cryopreservation harms acrosome morphology, motility, and enzyme activity in sperm (Sun et al., 2021b). Freezing affects the acrosomal membrane and induces pre-acrosomal reactions (Khan et al., 2021). Acrosome integrity in this study was higher compared to values obtained in previous studies including 22.8-30.46% using Sasaki diluent (Ruiz et al., 2024) and 35.16-53.16% with Lake diluent (Sun et al., 2021a). Freezing caused a slight increase in DNA damage as reported by Gliozzi et al. (2011) which recorded low sensitivity of chicken sperm to DNA fragmentation during the freezing process. Post-thawing sperm DNA damage in this study was lower than the results of previous studies using Lake diluent, namely 8-13.6% (Masoudi et al., 2021) and 19.82% (Gliozzi et al., 2011).

The cryopreservation process caused a decrease in mitochondrial functionality in this study. Post-thawing sperm ROS accumulation causes damage to the mitochondrial structure, changes in membrane potential difference and respiratory function, as well as decreased adenosine triphosphate (ATP) production and sperm motility (Fang et al., 2014; Słowińska et al., 2018; Zhang et al., 2021; Song et al., 2024). Abnormal ROS concentrations can disrupt the relationship between electron transport and oxidative phosphorylation leading to loss of mitochondrial function (Marcantonini et al., 2022). Continuous accumulation of ROS in cells damages mt-DNA and causes mitochondrial dysfunction (Song et al., 2024).

The addition of sorbitol and combination with BHT effectively maintained post-thawing spermatozoa mitochondrial functionality. This may be due to the ability of the treatment to maintain plasma membrane integrity thereby limiting the entry of excessive amounts of calcium into cells. Mitochondria actively contribute to cellular calcium buffering, but excess accumulation beyond physiological requirements leads to the opening of the mitochondrial permeability transition pore (mPTP), triggering apoptotic or necrotic cell death (Matuz-Mares et al., 2022). Calcium

accumulation can disrupt mitochondrial function, causing a decrease in ATP production as well as an increase in the release of ROS (Santulli et al., 2015). Defects in spermatozoa mitochondrial function greatly disrupt energy production required for motility. This explains the low sperm motility in the treatment without sorbitol and BHT observed (Khaeruddin et al., 2024b).

Ultrastructural observations offer clearer insights into the condition of the sperm surface. The freezing process damaged the sperm at the head, midpiece, and tail region but the addition of sorbitol and BHT in the diluent mitigated this damage. The results are in line with several ultrastructural analyses of cryopreserved chicken sperm in previous studies which found acrosome disruption, neck damage, loss of plasma membrane in the head (Heng et al., 2022), reduced mitochondrial matrix density (Zong et al., 2023), as well as uneven distribution of plasma and mitochondrial sheaths (Chen et al., 2024). Severe damage occurred in the middle part after cryoprepression in the control treatment. The midpiece contains mitochondria which contribute to energy metabolism (Blesbois, 2018), crucial for the progressive movement of spermatozoa (Sangani et al., 2017). The ROS can attack the plasma membrane and sperm mitochondria leading to dysfunction, release of cytochrome C into the cytoplasm, and initiation of cell apoptosis (Zhang et al., 2021).

Damage to sperm structure after freezing may be caused by ice crystallization, oxidative stress, heat shock, and osmotic shock (O'Neill et al., 2019). Poultry sperm have a higher amount of polyunsaturated fatty acids in the plasma membrane than several mammalian species (Najafi et al., 2020), increasing susceptibility to damage during the freezing process (Zong et al., 2023). An imbalance between the cellular antioxidant defense system and the production of ROS during freezing triggers oxidative stress which is the main cause of damage to sperm (Peña et al., 2019).

The combination of sorbitol and BHT prevents damage to sperm structure by maintaining membrane integrity due to their properties as an extracellular cryoprotectant and antioxidant respectively. The plasma membrane is a semipermeable barrier that protects the extracellular environment (Ray et al., 2016). Therefore, an intact plasma membrane is crucial to protect cell organelles such as mitochondria, nucleus, and acrosome from damage by external factors. The ability of cryoprotectant materials to maintain the ultrastructure of chicken sperm during freezing was also reported by Heng et al. (2022).

CONCLUSION

In conclusion, the addition of sorbitol 2% and combination with butylated hydroxytoluene (BHT) 3 mM effectively maintained plasma membrane integrity, mitochondrial functionality, and surface ultrastructure of Gaga chicken sperm during cryopreservation. Further studies are needed to evaluate the effects of sorbitol and BHT in diluents on sperm fertility after artificial insemination.

DECLARATIONS

Acknowledgments

The author thanks the Center for Higher Education Funding (BPPT) and Indonesia Endowment Funds for Education (LPDP). The author also thanks the Integrated Research Laboratory, Brawijaya University.

Funding

This research was funded by the Center for Higher Education Funding (BPPT) and Indonesia Endowment Funds for Education (LPDP), with grant number 1184/j5.2.3./bpi.06/10/2021.

Authors' contributions

Khaeruddin conducted research, laboratory work, and data analysis, and wrote the first version of the manuscripts. Gatot Ciptadi conducted laboratory work. Muhammad Yusuf revised the manuscript. Suyadi conducted sampling. Muhammad Halim Natsir conducted the statistical analysis. Herry Agoes Hermadi conducted the methodology and approved the final revision. Sri Wahjuningsih conducted the methodology and sampling. All the authors read and approved the final version of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Availability of data and materials

All data of the current study are available upon reasonable requests from the authors.

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