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Short Communication: The use of lactose-astaxanthin to maintain the quality of Green junglefowl frozen semen

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Abstract. *Bebas W, Agustina KK. 2022. Short Communication: The use of lactose-astaxanthin to maintain the quality of Green junglefowl frozen semen. Biodiversitas 23: 5759-5764.* The Green junglefowl is Indonesian germplasm that is at risk of population decline. Therefore, efforts are needed to protect its existence and survival. Cryopreservation of spermatozoa in the form of frozen semen is required to preserve this germplasm. This research aims to determine the effect of lactose-astaxanthin in preventing DNA fragmentation and intact plasma membrane of Green junglefowl spermatozoa during the freezing process. A completely randomized design with a 2x4 factorial pattern, including two treatment groups and four dimethylsulfoxide (DMSO) concentration factors, was used. The treatment groups are T1 and T2, consisting of egg yolk phosphate diluent and egg yolk phosphate diluent added with a combination of 0.6% lactose-0.004% astaxanthin, respectively. The four DMSO concentration factors were 4%, 6%, 8%, and 10% respectively. Subsequently, each treatment was repeated four times. The DNA fragmentation and intact plasma membrane of spermatozoa were observed, and the data obtained afterward were analyzed for variance, followed by the Ducan test. The results showed that the addition of 0.6% lactose-0.004% astaxanthin with 6% DMSO had a significantly better effect ($P<0.05$) on the quality of post-thawing spermatozoa with 19.75±0.96% DNA fragmentation and 53.75±3.86% intact plasma membranes. Therefore, it is recommended that a combination of 0.6% lactose-0.004% astaxanthin at 6% DMSO concentration should be added to Green junglefowl semen during the freezing process to prevent DNA fragmentation and intact plasma membranes.

Keywords: DMSO, DNA, frozen semen, Green junglefowl, intact plasma membrane, lactose-astaxanthin

INTRODUCTION

The Green junglefowl (*Gallus varius*), which has a very high economic potential and exists only in Indonesia, is at risk of population decline (Bebas and Laksmi 2013; Desta 2019). Therefore, efforts are needed to protect its existence and survival. The animal and its crosses are in great demand as favorite birds due to the attractive feather color and distinctive sound. The risk of the Green junglefowl population decrease and habitat loss is accelerated by settlement development and the community's low economic condition (Bebas and Laksmi 2013). Its existence in Indonesia is comparable to that of the Indian Red junglefowl, which has a population decline due to habitat destruction, hunting, egg collection, and genetic hybridization with native chickens (Rakha et al. 2016).

Cryopreservation of spermatozoa in the form of frozen semen, which can be used as a genetic bank (Santoso et al. 2021), is required to preserve this germplasm (Oldenbroek and Windig 2022). The stages of the freezing process include storage, dilution, equilibration at 4^{o}C, gradual temperature reduction, freezing, and thawing (Iaffaldano et al. 2016, 2021). However, semen often experiences challenges during cryopreservation, especially in the freezing and thawing processes (Simonik et al. 2022). There is a massive release of water molecules from the cell during freezing (Jang et al. 2017). Furthermore, this increases the concentration of intracellular electrolytes and the formation of ice crystals, known as cold shock, which cause structural and biochemical damage to cells, affecting cell function (Prickett et al. 2015). It damages cell membranes, affects motility and abnormalities, and ultimately causes cell death (Solihati et al. 2017; Bebas et al. 2018).

Spermatozoa undergo a peroxidation process during freezing (Castro et al. 2016; Rizkallah et al. 2022). This is caused by free radicals, which are highly reactive and can damage the lipid of the plasma membrane (Asadi et al. 2017; Dutta et al. 2019). The lipid oxidation in spermatozoa's plasma membrane is toxic to cells and can cause damage to DNA (Bebas et al. 2016; Dutta et al. 2019).

Some substances, such as cryoprotectants, that work extracellularly and intracellularly can be added to the diluent to avoid the negative effect on the semen freezing process (Bebas et al. 2016; Bebas et al. 2018). Adding carbohydrates such as glucose, fructose, and lactose can act as an extracellular cryoprotectant and also as an energy source that maintains the quality of spermatozoa during storage (Hall et al. 2017; Saha et al. 2022). Previous research stated that the optimal addition of lactose to egg

yolk phosphate diluent to dilute domestic chicken semen was 0.6% (Bebas and Laksmi 2015)

Astaxanthin is a carotenoid group of antioxidants which is currently the strongest (Ambati et al. 2014; Nishida et al. 2021). Furthermore, it can tackle free radicals using "scavenges" (Dose et al. 2016; Nishida et al. 2021; Pereira et al. 2021). Previous research added astaxanthin to sperm dilution to maintain the quality of spermatozoa during storage (Indrawati et al. 2013; Bebas and Gorda 2020). Adding 0.004% of astaxanthin to egg yolk phosphate diluent is known to maintain the quality of domestic chicken sperm stored at a temperature of 3-5ºC (Indrawati et al. 2013).

DMSO, as an intracellular cryoprotectant, protects cells from the inside by balancing intra and extracellular osmolality, as well as modifying the surface structure of the ice crystals, hence, they don't become too sharp. Using it as an intracellular cryoprotectant positively affects the freezing process of Green junglefowl (Bebas and Laksmi 2014) and pig semen (Bebas and Gorda 2020).

This research aims to determine the effectiveness of the combination of 0.6% lactose-0.004% astaxanthin and DMSO concentration in preventing DNA fragmentation and intact plasma membranes of Green junglefowl spermatozoa involved in the freezing process.

MATERIALS AND METHODS

Ethical clearance

This research with Decree No. B/186/UN14.2.9/ PT.01.04/2022 was approved by the ethics committee of the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia.

Research design

A completely randomized design with a 2x4 factorial pattern, including two treatments and four factors, was used. The treatment groups are T1 and T2, consisting of egg yolk phosphate diluent and egg yolk phosphate diluent with the addition of 0.6% lactose- 0.004% astaxanthin, respectively. Meanwhile, the four DMSO concentration factors are 4%, 6%, 8%, and 10%. Finally, the parameters observed were DNA fragmentation and intact plasma membrane of spermatozoa.

Research sample

The sample used was eight male Green junglefowl aged 1.5-2 year with a weight of 1250 to 1345g. They were kept in cages made of bamboo with a diameter of 50cm and a height of 75cm. These birds were fed a mixture of cracked corn, brown rice, ground mung beans, and unhulled rice in a ratio of 1:1:1:1. Furthermore, the Green junglefowl are given different food to taste crickets, and drinking water was given ad libitum, every three days anti-stress drugs were added to maintain their health.

Semen collection

Semen was collected by the massage method introduced by Burrows and Quinn (Yadav et al. 2019). The spermatozoa accumulated from the eight males were homogenized and evaluated for their quality macroscopically and microscopically.

Semen diluent manufacturing

The egg yolk phosphate diluent was prepared by adding 10% egg yolk into phosphate buffer with 80 mg/L of the antibiotic kanamycin (Khasanova et al. 2019). Meanwhile, the combination of lactose 0.6%-astaxanthin 0.004% was made by adding 0.6g of crystalline lactose and 0.004g of astaxanthin into 100 mL of egg yolk phosphate diluent.

Production of egg yolk phosphate diluent

A total of 0.4024g NaH_2PO_4 50 mM and 0.9228g $Na_2HPO_4.H_2O$ 50 mM was dissolved in 500 mL of distilled water (phosphate diluent pH 7). A total of 10 mL of fresh egg yolk was poured into 90 mL of the phosphate diluent. Addition of 0.6% Lactose and 0.004% Astaxanthin to egg yolk phosphate diluent. A total of 0.004g of Astaxanthin and 0.6g of Lactose monohydrate were dissolved in 100 mL of egg yolk phosphate diluent.

The astaxanthin used were 3,3′-Dihydroxy-β-carotene-4,4′-dione, trans-Astaxanthin; Sigma-Aldrich (SML0982) 97% (HPLC), derived from *Blakeslea trispora*. Meanwhile, the lactose monohydrate used is milk sugar for microbiology (CAS 10039-26-6, pH 4.0-6.5).

Semen dilution treatment

Group T1: Use egg yolk phosphate diluent only.

Group T2: Use egg yolk phosphate with 0.6% lactose-0.004% astaxanthin.

The treatment combinations were prepared according to the factorial design by adding 4%, 6%, 8%, and 10% DMSO, respectively. Semen dilution in each treatment combination was conducted at a room temperature 18ºC. Finally, each mL of the spermatozoa contains 150 million progressive motile cells.

Semen packaging, freezing, and storage

Using an automatic filling and sealing machine (MPP Uno, automatic filling and sealing machine for semen straws. REF: 13017/0000 (Minitube, Germany), the diluted semen was packed in 0.25 mL straws equilibrated at 4ºC for 2 hours (Cold handling cabinet, Minitub, Germany). The freezing process is conducted using a special tool, with a gradual decrease in temperature from 4ºC to -120ºC for 10 minutes. Furthermore, the semen was immersed in liquid N_2 for freezing and was stored in a container (-196ºC). Finally, after one week of storage, the semen's quality was evaluated by thawing in warm water (37ºC) for 15 seconds until melted.

DNA fragmentation check

The DNA fragmentation was examined using Terminal Deoxynucleotidyl Transferase (TdT) nick-end labeling (TUNEL) test (Sharma et al. 2013; Bebas et al. 2016). The TUNEL method was used to identify single and double DNA strands damaged or truncated by labeling free OH groups. Spermatozoa with damaged nuclei appear brown, while those with intact DNA appear blue. The workings are

performed according to the Tunel-Biotin-Avidin-HRP Protocol (DAB) Kit.

Solutions and Reagents used were: A. 10X Phosphate Buffered Salin (PBS) consist of 10.9g Na_2HPO_4, 3.2 g NaH_2PO_4, 90g NaCl, 1000mL Distilled water with pH 7.4; B. 3% Hydrogen Peroxide consist of 10 mL 30% H_2O_2 to 90 mL PBS or methanol; C. TdT Buffer (30 mM Tris base, 140 mM sodium cacodylate, 1 mM cobalt chloride) consist of Reaction buffer, 40 uL 5x (Boehringer Mannheim), 8 uL $CoCl_2$ (25 mM, Boehringer Mannheim) and 160 uL Distilled water; D. TdT Enzyme Mixture consist of 2.0 uL TdT enzyme (Boehringer Mannheim), 2.0 uL Biotin-16-dUTP (Boehringer Mannheim), 500 uL TdT buffer (Made above); E. Stop Wash (300mM NaCl, 30mM Sodium Citrate) consist of 30 mL 1.0 M NaCI, 3 mL 1.0 M Sodium citrate and 67 mL Distilled water; F. ABC Reagent: Depending on sensitivity and morphological requirements, there are varieties of ABC kits and reagents can be selected from. The most commonly used one is VECTASTAIN ABC Kit from Vector Laboratories; G. DAB Reagent: Procedure, Deparaffinize sections in xylene for 3x5min; Hydrate with 100% ethanol for 2x5min; Hydrate with 95% ethanol for 2x5min; Rinse in distilled water; Hydrogen Peroxide: incubate sections in 3% H_2O_2 in PBS (or methanol) for 10-15 minutes to block endogenous peroxidase activity; Rinse in PBS for 2x5min; Incubate sections in TdT buffer for 10 minutes; Incubate sections in TdT enzyme mixture for 3 hours at 37ºC; Rinse in stop wash solution for 10 minutes; Rinse in PBS for 3x5min; ABC: incubate sections with ABC complex or HRP-streptavidin reagent in PBS for 30 minutes at room temperature; Rinse in PBS for 3x5min; DAB: incubate sections with DAB solution for 2-10 minutes; Rinse in PBS for 3x5min; Counterstain with hematoxylin if desire; Rinse in distilled water 2x5min; Dehydrate through 95% ethanol for 5min, 100% ethanol for 2x5min; Clear in xylene for 2x5min; Coverslip with mounting medium. Results: DNA damaged nuclei (brown) and Normal nuclei (blue).

Intact plasma membrane examination

The intact plasma membranes were examined using the Hypoosmotic swelling test (HOS) method (Prochowska et al. 2022). The hypoosmotic solution consisted of 0.9g of fructose + 0.49g of sodium citrate dissolved in distilled water to reach a volume of 100 mL. Subsequently, 20 mL of it was added with 0.2 mL of semen, sealed in a purified plasma membrane until homogeneous, and incubated at 37°C for 45 minutes. Thin smear preparations were made on an object glass and evaluated with a light microscope at 400x magnification for a minimum of 200 spermatozoa. The sperm cells with intact plasma membranes are characterized by coiled or bulging tails, while straight tails indicate those that are damaged.

Data analysis

The data obtained were analyzed by analysis of variance and continued with the Ducan test on the SPSS 25 for Windows program.

RESULTS AND DISCUSSION

Fresh semen evaluation

The semen was collected in one test tube, homogenized, and evaluated for its macroscopic and microscopic quality, as presented in Table 1.

The evaluation results showed that the quality of Green junglefowl semen was very suitable for use and can be further processed to freeze. The semen quality inspection above was consistent with previous research by Bebas and Laksmi (2015) as well as Bebas et al. (2015).

Evaluation of post thawing semen.

The results showed that adding 0.6% lactose-0.004% astaxanthin to egg yolk phosphate diluent resulted in a significantly better quality of post-thawing semen ($p<0.05$). The addition of various concentrations of DMSO in egg yolk phosphate diluent also had a significant effect ($p<0.05$) on the quality of post-thawing semen. Finally, the impact of these treatments on DNA fragmentation and intact plasma membranes is shown in Tables 2 and 3.

Figure 1 shows that adding DMSO at a concentration of 6% in the diluent produces the least post-thawing spermatozoa DNA fragmentation of 19.75±0.96. Furthermore, the description of sperm cells with DNA fragmentation is shown in Figure 2.

Figure 3 shows that adding DMSO at a concentration of 6% produced the best results, where the intact plasma membrane of post-thawing spermatozoa has the highest value of 53.75±3.86%. The normal spermatozoa with intact plasma membrane can be shown in Figures 4 and 5, respectively.

Table 1. Quality of fresh Green junglefowl semen

Green junglefowl semen quality	
Volume (mL)	2.20
pH	7.1
Color	White
Smell	Typical
Thickness	Thick
Concentration (10^9/mL)	2.97
Progressive movable sperm (%)	85
Live spermatozoa (%)	90
Abnormalities (%)	8
Total spermatozoa (10^9)	6.534
Progressively movable sperm total (10^9)	5.5539

Table 2. DNA fragmentation of Green junglefowl spermatozoa post thawing

Parameter	Treatment	Group	Average±SD
DNA fragmentation (%)	Diluent (Control)	DMSO 4%	33.50±1.29[a]
		DMSO 6%	26.25±0.96[b]
		DMSO 8%	41.25±1.50[c]
		DMSO 10%	48.00±1.41[d]
	Diluent plus 0.6% lactose-0.004% astaxanthin	DMSO 4%	24.75±0.96[e]
		DMSO 6%	19.75±0.96[f]
		DMSO 8%	29.75±0.96[g]
		DMSO 10%	41.75±1.50[h]

Description: Different superscript letters in the direction of the column indicate a significant difference (P<0.05)

Table 3. The intact plasma membrane of post-thawing Green junglefowl spermatozoa

Parameter	Treatment	Group	Average± SD
Intact plasma membrane (%)	Diluent (control)	DMSO 4%	35.00±3.92[a]
		DMSO 6%	47.75±3.77[b]
		DMSO 8%	24.25±4.19[c]
		DMSO 10%	7.00±2.58[d]
	Diluent plus 0.6% lactose - 0.004% astaxanthin	DMSO 4%	39.75±2.50[e]
		DMSO 6%	53.75±3.86[f]
		DMSO 8%	30.50±4.65[g]
		DMSO 10%	9.00±2.16[h]

Description: Different superscript letters in the direction of the column indicate a significant difference (P<0.05)

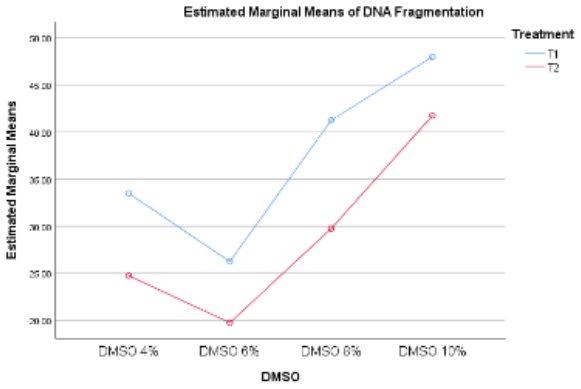

Figure 1. Effect of DMSO treatment and concentration on DNA fragmentation of post-thawing Green junglefowl spermatozoa; Blue line (T1): Egg yolk phosphate diluent; Red line (T2): Egg yolk phosphate diluent plus 0.6% lactose-0.004% astaxanthin

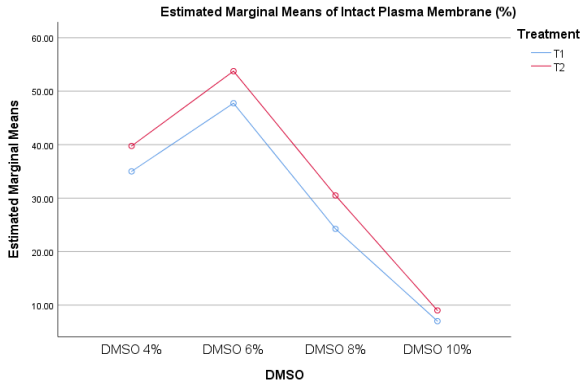

Figure 3. Effect of DMSO treatment and concentration on intact plasma membranes of post-thawing Green junglefowl spermatozoa; Blue line (T1): Egg yolk phosphate diluent; Red line (T2): Egg yolk phosphate diluent plus 0.6% lactose-0.004% astaxanthin

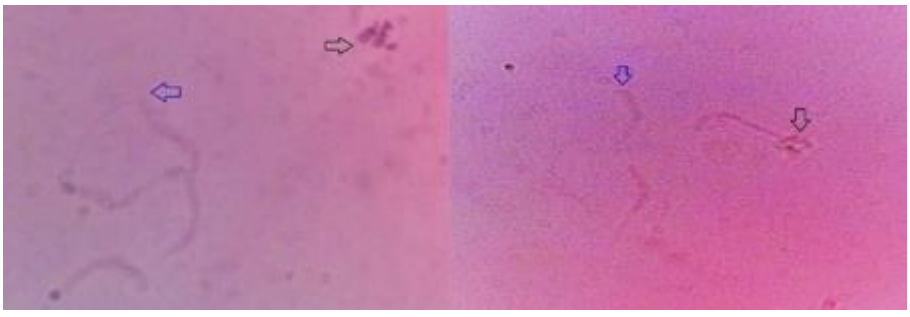

Figure 2. Spermatozoa with DNA fragmentation (1000X magnification); Black arrow: Spermatozoa with DNA fragmentation; Blue arrow: Spermatozoa without DNA fragmentation

Figure 4. Normal spermatozoa shape. (400X Magnification)

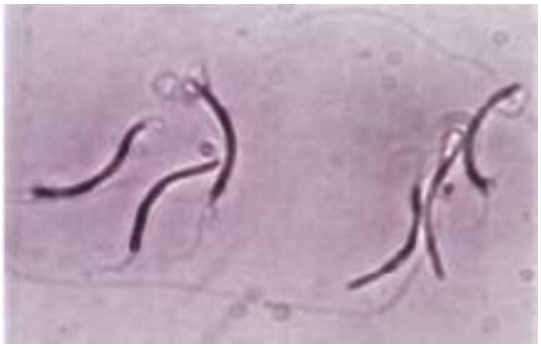

Figure 5. Spermatozoa with intact plasma membrane marked circular tail and swelling (400X magnification)

Discussion

Adding 0.6% lactose-0.004% astaxanthin to egg yolk phosphate diluent reduced DNA fragmentation and increased the percentage of intact spermatozoa plasma membranes (Figures 1-3). Adding 0.6% lactose-0.004% astaxanthin to egg yolk phosphate diluent for freezing Green junglefowl sperm had a positive effect on the post-thawing quality of the sperm. This formulation was used to maintain the semen quality during storage at 5ºC (Bebas et al. 2016).

The role of lactose or other types of carbohydrates in semen storage is to reduce damage to cell plasma membranes during the process of cooling, freezing, and thawing (Bebas et al. 2018). Therefore, carbohydrates increase the fluidity of the plasma membrane before freezing (Stanishevskaya et al. 2021). Stanishevskaya et al. (2021a), as well as Bebas and Laksmi (2015), stated that lactose plays a role in replacing the water position on the surface of the cell plasma membrane, which is directly related to the diluent. It can interact directly with the polar phospholipid central group during freezing and reduce the interaction of van der Walls bonds between the carbon chains (Iqbal et al. 2018). Meanwhile, adding carbo-hydrates, either monosaccharides or disaccharides, to the diluent for semen freezing is helpful as an extracellular cryoprotectant essential in maintaining membrane flexibility (Athurupana et al. 2015; Stanishevskaya et al. 2021; Stanishevskaya et al. 2021a).

The addition of 0.004% astaxanthin to the diluent acts as an antioxidant capable of intact plasma membranes. This was conducted to cope with cell membrane lipid peroxidation during the freezing and post-thawing processes (Lee and Kim 2018: Guo et al. 2021). Astaxanthin is currently the most potent antioxidant to combat free radicals by scavenging O_2^-, which reacts directly with peroxyl radicals (Dose et al. 2016; Brotosudarmo et al. 2020). The esterification ability and antioxidant activity are higher and more polar due to its unusual molecular structure, which contains two carbonyl groups, two hydroxy groups, and eleven conjugated double bonds (Pérez-Gálvez et al. 2020; Pereira et al. 2021). Furthermore, astaxanthin can protect and fight lipid membrane peroxidation, such as polyunsaturated fatty acids and proteins, DNA fragmentation, as well as the ultraviolet light effect. It plays an essential role in the immune system process (Bebas and Gorda 2020; Pereira et al. 2021).

Oxidative stress can increase DNA fragmentation, causing an increase in the mutagenesis content of 8-hydroxy-2-deoxyguanosine (8-OH-2-dG) (Sudharshan and Dyavaiah 2021). Astaxanthin can prevent or reduce the oxidation of guanosine (Zhu et al. 2021). Subsequently, adding lactose-astaxanthin combination to egg yolk phosphate diluent for storage of Green junglefowl semen at 5ºC maintained progressive motility and reduced DNA fragmentation (Bebas et al. 2016). This can also improve the quality of Gembrong goat frozen semen (Bebas et al. 2018).

DMSO, as an intracellular cryoprotectant, can protect cells from the inside by balancing intra and extra-cell osmolality and modifying the surface structure of ice crystals, hence, they don't become too sharp (Whaley et al. 2021; Sreter et al. 2022). The effectiveness of using DMSO for freezing Green junglefowl and pig semen was reported by Bebas and Laksmi (2014) and Bebas and Gorda (2020), respectively.

This study concludes that a combination of 0.6% lactose-0.004% astaxanthin and 6% DMSO concentration to egg yolk phosphate diluent produced the best quality of post-thawing Green junglefowl semen with 19.75% DNA fragmentation and 50.75% intact plasma membrane.

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