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Effect of Glutathione Supplementation in Liquid Semen Diluent During Cold Storage on Sperm Membrane Structure in Ongole Crossbred Bull

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ABSTRACT

Damage to bovine spermatozoa membranes results from factors such as oxidative stress, high temperatures, extreme pH levels, and exposure to chemicals or infections. The present study aimed to explore the potential use of glutathione in liquid semen extenders during cold storage to protect the membrane structure of Peranakan Ongole (PO) crossbred bull semen. Semen samples were collected twice weekly from two PO crossbred bulls aged 2-3 years. Semen that met the quality standards was diluted with a red fruit (*Pandanus conoideus Lamk*) extract extender, supplemented with glutathione at various concentrations per 100 mL of the total volume extender. Sperm membrane evaluation included membrane integrity assessed by the hypoosmotic swelling test (HOST), sperm capacitation, and acrosome reaction assessed by chlortetracycline fluorescence staining. The treatments consisted of red fruit extract extender with 15% egg yolk (P0, control), red fruit extract extender with 15% egg yolk and 0.75 mM glutathione (P1), red fruit extract extender with 15% egg yolk and 1 mM glutathione (P2), and red fruit extract extender with 15% egg yolk plus 1.25 mM glutathione (P3). The present results indicated that Group P1 performed superior than other treatment groups. This was evidenced by higher spermatozoa membrane integrity and a higher proportion of non-capacitated spermatozoa, while the proportions of capacitated spermatozoa and acrosome reaction remained low. The extender formulation containing red fruit extract, 15% egg yolk, and 0.75 mM glutathione-maintained acrosome status above 50% after six days of cold storage. Therefore, the combination of red fruit extract, 15% egg yolk, and 0.75 mM glutathione was identified as the optimal formulation for liquid semen preservation, meeting the necessary conditions for practical use in artificial insemination programs in the field.

Keywords: Acrosome reaction, Glutathione, Membrane integrity, Red fruit extract

INTRODUCTION

Increasing the population and productivity of local cattle can be achieved through reproductive technologies, such as artificial insemination (AI). Artificial insemination is a reproductive technology introduced to commercial breeders to improve the genetic quality of livestock and increase beef production (Ervandi et al., 2020). The success of AI is influenced by the quality of semen produced by the cattle (Yekti et al., 2017). Semen quality can decrease during cryopreservation (preservation/storage) due to thermal cold shock, mechanical formation of intracellular ice crystals, chemical factors (diluent components), and osmotic stress (Khan et al., 2021). Semen diluents are essential for reducing the risk of decreased semen quality during processing and post-processing (Dzulqarnain et al., 2022). Commercial diluents are considered more practical as diluents (Ervandi et al., 2023). Previous studies have identified several diluents for liquid semen, using natural ingredients such as coconut water, carrot juice, tomato juice, egg yolk, honey, and guava filtrate (Sumadiasa et al., 2015; Malik et al., 2018; Astuti, 2018; Marawali et al., 2019). However, the resulting semen quality typically lasts for three to four days.

Based on previous studies, alternative natural liquid semen diluents, such as a combination of red fruit extract and coconut water, have not been able to maintain semen quality for long periods and are ineffective at protecting sperm membranes at low temperatures. Consequently, the quality and fertility of liquid semen decline with increasing storage time, partly due to the accumulation of excessive free radicals (Ervandi et al., 2023). Excessive free radicals can damage the sperm plasma membrane (Agung et al., 2023). During this process, spermatozoa undergo peroxidation, which generates free radicals, such as hydroxyl (-OH) and singlet oxygen ($^1O_2$; Bansal and Bilaspuri 2011; Park and Yu 2017). These radicals are highly reactive and potentially induce lipid peroxidation in the plasma and acrosome membranes (Douard et al., 2003). Lipid oxidation in the plasma membrane produces malondialdehyde (MDA), a marker of toxic free radicals, which reduces motility and causes DNA damage (Dutta et al., 2019). Wijayanti et al. (2023) indicated that the initial reactions of free radicals, if left uncontrolled, may initiate a continuous process that could potentially impair most

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or all of the spermatozoa plasma membrane. Therefore, the urgency of the present study highlighted the necessity of developing effective methods to preserve the quality of liquid semen at low temperatures by incorporating antioxidants into the semen extender, such as vitamin E, catalase enzyme, and glutathione, which have been identified to preserve semen quality (Telnoni et al., 2023). Although both compounds exhibited considerable antioxidant capacity, for instance, vitamin E has limited water solubility, resulting in uneven distribution within the semen extender. The catalase enzyme, being a large protein, is susceptible to denaturation at extreme temperatures during freezing and semen storage (Câmara et al., 2016; Ahmad et al., 2021; Wadhwa et al., 2024). As an alternative, glutathione, an endogenous antioxidant, exhibits promising potential for use in semen extenders (Ahmad et al., 2021). Glutathione is a primary antioxidant that prevents the formation of new free radicals (Ansari et al., 2021). Glutathione plays a crucial role in the cellular biochemical system as a primary antioxidant, detoxifying agent, and vital regulator of redox signaling. Its function is essential for maintaining cellular oxidation and reduction balance, supporting mitochondrial function, and preserving protein structural stability, particularly through the S-glutathionylation mechanism. The biological functions of glutathione are carried out by several specific enzymes, such as glutathione peroxidase, glutathione reductase, and glutaredoxin, which collectively contribute to the regulation of oxidation and reduction homeostasis and the response to oxidative stress (Carriço et al., 2023; Chai and Mieyal, 2023; Chen et al., 2024). Munsi et al. (2007) reported that glutathione added to semen extenders significantly increases sperm motility and reduces acrosomal damage in bull semen stored at low temperatures. Antioxidants are compounds that can slow or prevent damage to the spermatozoa plasma membrane caused by free radicals by counteracting free radical activity or breaking the chain of oxidation reactions caused by free radicals (Zou et al., 2021). Solihati et al. (2018) and Zou et al. (2021) suggested that the use of glutathione in dilution media is expected to reduce the emergence of free radicals that can damage the plasma membrane. Adding glutathione or enhancing related enzymatic activities in liquid semen extenders has been shown to improve sperm viability and motility after dilution, reduce oxidative damage to sperm membranes and DNA, and extend the shelf life of liquid semen (Zhu et al., 2023).

Damage to the bovine spermatozoa membrane can result from different factors, including oxidative stress, high temperature, extreme pH, and exposure to chemicals or infections (Younus et al., 2024). Membrane damage affects the integrity of the spermatozoa membrane, which can alter its ion system (Xue et al., 2025). Disruption of calcium ion transport across the cell membrane results in elevated intracellular calcium levels, which can induce capacitation and the acrosome reaction in spermatozoa (Maleki et al., 2023). Additionally, oxidative stress plays a crucial role in membrane damage by altering lipid components in the plasma membrane, altering membrane fluidity, and disrupting its functional integrity (Solihati et al., 2018). Xue et al. (2025) indicated that damage to the spermatozoa membrane may impair their ability to reach the oocyte, potentially leading to fertilization failure. Therefore, during the assessment of the quality of liquid semen from Peranakan Ongole (PO) crossbred bulls, the membrane structure parameter is an essential aspect to observe, as the membrane structure test can evaluate the permeability of the plasma membrane in maintaining spermatozoa physiology. Additionally, the intact acrosome membrane (IAM) cap test assesses the potential of spermatozoa to penetrate the zona pellucida during fertilization, through the acrosome reaction (Susilawati, 2011). Therefore, the present study aimed to determine the structure of the semen membrane of PO bulls after glutathione supplementation in liquid semen diluent during cold storage.

MATERIALS AND METHODS

Ethics approval

Ethics approval has been granted by the Ethics Committee of the Faculty of Science and Technology, Muhammadiyah University of Gorontalo, Gorontalo, Indonesia, in accordance with ethical guidelines regarding responsible behavior in the use of Ongole crossbred (PO) bulls in animal research. Ethics approval number is 081/FST-UMGO/V/2024.

Materials and equipment

The bovine semen was collected from two PO bulls aged approximately two to three years old at the Integrated Field Laboratory, Muhammadiyah University of Gorontalo, Indonesia. The semen used met the criteria of mass motility ≥++ and individual motility ≥ 70%, collected twice weekly using the artificial vagina method, and included ingredients, such as red fruit extract extender, egg yolk, glutathione, Diazabicyclooctane (DABCO) solution, Chlortetracycline (CTC) staining fixative solution, CTC staining dye solution, aluminum foil, fructose, penicillin, aqua bidestilata, 70% alcohol, 3% NaCl, and eosin-nigrosin. The equipment utilized included Olympus CX23 multimedia (Olympus corporation, Japan) microscope, epifluorescence microscope OPTIPHOT-2 using a UV-2A filter (Nikon Corporation, United Kingdom), centrifuge LC-8 C3100 (Benchmark Scientific Corporation, USA), cover slips, microscope slides,

thermometer, heater, pH indicator, water bath, magnetic stirrer, hemocytometer, refrigerator, Eppendorf tube, analytical balance, scissors, micropipettes, blue tips, yellow tips, Erlenmeyer flasks, test tubes, glass beakers, and glass stirrers.

Semen evaluation

The collected semen from a single male was subjected to both macroscopic and microscopic examinations. The macroscopic examination involved assessing volume, pH, consistency/viscosity, color, and odor. Sperm quality, including mass motility, individual motility, spermatozoa concentration, abnormalities, membrane integrity, and acrosome status, was evaluated microscopically using an Olympus CX23 microscope at 400× magnification (Susilawati 2011).

Liquid semen diluent with red fruit extract

The red fruit extract diluent was weighed (100 g), added to 300 mL of distilled water, blended for 5 minutes, and then left to settle for 2.5 hours. Afterward, the mixture was filtered using a filter cloth. This filtering process was repeated twice. The second filtering process used filter paper to obtain the red fruit extract. Then, fructose at 10 and 20 mg/mL, penicillin antibiotic at 1 mg/mL, sorbitol at 1000 µg/L, streptomycin sulfate at 1 mg/mL, and gentamicin at 0.05 µg/mL were added. After the red fruit extract diluent was prepared, egg yolk was added for subsequent use as a membrane protector. Egg yolk (15%) was added to the red fruit extract diluent (85%) to obtain a final extender concentration of 100%. The mixture was stirred for 30 minutes, then centrifuged at 1500 rpm for 30 minutes. Red fruit extract diluent supplemented with 15% egg yolk was stored at 3-5°C in a refrigerator. Subsequently, the mixture was prepared for use in the following stages of the study (Ervandi et al., 2023). For the test group, glutathione was added to the red fruit extract extender diluent at different concentrations according to treatments (Maleki et al., 2023).

Storage duration

Following dilution, semen samples were aliquoted into test tubes based on the assigned storage duration treatments. The tubes were placed in a test tube rack and stored at 5°C in a refrigerator. At each designated storage interval, semen samples were assessed for sperm motility, viability, abnormality, and membrane integrity (Syarifuddin et al., 2012).

Semen preservation process

Semen samples that were diluted with an extender and stored in a refrigerator at 5°C for several days were then evaluated for mass motility, individual motility, sperm concentration, abnormalities, membrane integrity, and acrosome status (Swarna et al., 2023).

Study design

The present study employed a randomized block design (RBD) with four replicates and 10 replications. Grouping was based on different semen collection times. The treatments included red fruit extract extender with 15% egg yolk (P0, control), red fruit extract extender with 15% egg yolk and 0.75 mM glutathione (P1), red fruit extract extender with 15% egg yolk and 1 mM glutathione (P2), and red fruit extract extender plus 15% egg yolk plus 1.25 mM glutathione (P3).

Sperm membrane integrity examination

Membrane integrity observation

Sperm cell membrane integrity was tested using the hypoosmotic swelling test (HOST). One milliliter of a hypoosmotic solution at 125 Osm/L, which was made from 0.31 g of sodium citrate and 0.565 g of fructose, dissolved in 50 mL of distilled water, was added to 100 µl of spermatozoa. The solution was then incubated at 37°C for 30 minutes. A smear was then prepared and observed under a light microscope (Olympus CX23 Multimedia, Japan) at ×400 magnification. Coiled or swollen tails characterized spermatozoa with IPM, while damaged ones exhibited straight tails (Susilawati 2011). Purnawan et al. (2023) stated that sperm quality analysis, including the assessment of plasma membrane integrity, was conducted by examining at least 200 cells per sample to ensure statistical reliability and a representative evaluation of the sperm population. Krishna et al. (2024) stated that the membrane integrity value of spermatozoa can be calculated using the following formula.

Membrane integrity (%) = (number of spermatozoa with intact plasma membranes / total number of spermatozoa observed) × 100

(Formula 1)

Observing sperm capacitation and the acrosome reaction

The capacitation of fresh spermatozoa can be observed using CTC staining. The CTC staining involves three final reagents, including DABCO solution, CTC fixative solution, and CTC dye solution. The procedure was performed

according to Susilawati (2011) with the following modifications. A total of 100 µL of semen (fresh and treated) was placed in an Eppendorf tube covered with aluminium foil, then 100 µL of CTC dye was added and vortexed for one minute. Then, 8 µL of CTC fixative was added, and the mixture was vortexed for one minute to homogenize. Then, 10 µL of the solution was taken and placed on a glass slide. Subsequently, 10 µL of DABCO was added, and the combination was carefully mixed. The slide was then covered with a coverslip, then covered with thick tissue paper and pressed carefully. The edges of the coverslip were sealed with nail polish, and the coverslip was placed in a water bath at 37°C. The mixture was left for approximately three minutes (Susilawati, 2011). Observations were conducted utilizing an Epifluorescence microscope OPTIPHOT-2 equipped with a UV-2A filter, examining 100 spermatozoa from a single field of view slide (Susilawati, 2011; Ervandi et al., 2013). The CTC staining results indicated that spermatozoa with completely bright-stained heads were non-capacitated. Conversely, bright staining on the upper part of the head indicated sperm capacitation (Krishna et al., 2024).

Statistical analysis

The current data were first transformed and subsequently analyzed using Analysis of Variance (ANOVA) to evaluate differences among treatments, followed by Duncan's post hoc test. A significance level of $p < 0.05$ was considered statistically significant, while $p < 0.01$ was regarded as highly significant (Sudarwati et al., 2019).

RESULTS AND DISCUSSIONS

Characteristics of fresh semen from Ongole crossbred

The fresh semen from Ongole crossbred bulls used in the present study had a motility of above 70%. The quality and macroscopic examination of the collected fresh semen samples are presented in Table 1.

Macroscopic examination of fresh semen is shown in Table 1. The average semen volume of Ongole crossbred bulls was 6.14 ± 1.16 mL, which was higher than the results of Yekti et al. (2017) and Mila et al. (2021), which were 4.17 ± 1.17 mL and 4.6 ± 1.51 mL, respectively. Suyadi et al. (2020) reported that the volume of semen in PO bulls in each ejaculation ranged from 5 to 10 mL, and the semen color was cream, which was regarded as an acceptable finding. Susilawati (2013) stated that normal semen is yellowish-white (cream) or milky-white, and the degree of turbidity depends on spermatozoa concentration. The degree of acidity (pH) in Ongole crossbred bulls' semen was 6.40 ± 0.10, lower than the findings of Yekti et al. (2017) and Dzulqarnain et al. (2022) at 6.63 ± 0.08 and 6.42 ± 0.23, respectively. The fresh semen from the Ongole crossbred bulls had a thick consistency. Ismaya (2014) indicated that semen consistency can be assessed by gently shaking the tube containing the semen, indicating a sperm concentration of 1,000 to 2,000 million/mL. The optimal pH of bovine semen ranges from 6.45 to 6.57, with a concentration of 1223.7 to 1961.8 million spermatozoa per milliliter, and the ideal individual motility is around 70%, along with a mass movement rating of 2+ (Arif et al., 2020; Wadhwa et al., 2024; Xue et al., 2025). The mass motility of Ongole crossbred bulls in the present study was 2+, while the individual motility was 76.40 ± 2.54. These findings are higher than those reported by Yekti et al. (2017) for Ongole bulls, with a value of 71.67 ± 2.58. The fresh Ongole bull semen demonstrated high quality, with both viability and abnormality percentages within the normal range. The current results align with the findings of Suyadi et al. (2014; 2020), who reported that fresh semen from Ongole bull can achieve up to 80% viability and maintain an average abnormality rate of 12-18% when stored under optimal conditions. The fresh semen samples from PO bulls exhibited characteristics that complied with the quality requirements for further processing.

Table 1. Fresh semen quality from Ongole crossbred bull

Parameters	Average ± Standard deviation
Macroscopic	
Volume (ml)	6.14 ± 1.16
pH	6.40 ± 0.10
Scent	Distinctives
Consistency	Thick
Color	Cream
Microscopic	
Individual motility (%)	76.40 ± 2.54
Viability (%)	92.10 ± 2.05
Abnormality (%)	2.81 ± 0.33
Sperm concentration (10^7/ml)	1351.09 ± 16.70
Mass motility	2+

Individual motility of Ongole crossbred semen

The average individual motility of PO bulls' semen after treatment is presented in Table 2. The individual motility of PO bulls demonstrated the highest durability in diluents P1 and P2, lasting up to six days with values of 44.21 ± 1.19% and 40.36 ± 1.32%, respectively, compared to P0 and P3. The present results indicated a significant difference ($p < 0.01$) in the individual motility of PO bulls on days six to eight of storage. The Least Duncan's post hoc test indicated that the P1 and P2 diluent treatments yielded the best results, with values of 44.21 ± 1.19% and 40.36 ± 1.32%, respectively, during the six days of storage. Red fruit extract diluent with 15% egg yolk supplemented with 0.75 mM glutathione (P1) demonstrated superior results compared to P0 (control), P2, and P3. This is attributable to the composition of P1, which maintains an optimal balance between antioxidant effects and sperm quality stability. Glutathione at 0.75 mM effectively improved protection of sperm cell membranes from oxidative damage without inducing osmotic stress or disrupting the redox equilibrium, concerns typically associated with higher glutathione concentrations, such as in 1 mM (P2) and 1.25 mM (P3).

Table 2. Mean motility of individual Ongole crossbred bulls with various levels of glutathione diluent during cold storage

Treatment	Mean motility (%) ± Standard deviation								
	D-1	D-2	D-3	D-4	D-5	D-6	D-7	D-8	D-9
P0 (RFE plus 15% EY)	70.31 ± 1.38^{a}	60.15± 1.29^{a}	58.76 ± 1.20^{a}	45.11 ± 0.19^{a}	40.14 ± 0.17^{a}	39.10 ± 0.15^{b}	35.16 ± 1.11^{b}	18.51 ± 1.10^{b}	14.20 ± 0.12^{a}
P1 (RFE plus 15% EY plus 0.75 mM GSH)	70.34 ± 1.14^{a}	69.16 ± 1.32^{a}	65.17 ± 1.24^{a}	60.57 ± 1.28^{a}	55.19 ± 1.20^{a}	44.21 ± 1.19^{a}	38.29 ± 1.11^{a}	23.41 ± 0.09^{a}	19.21± 0.00^{a}
P2 (RFE plus 15% EY plus 1 mM GSH)	66.13 ± 1.31^{a}	60.54 ± 1.56^{a}	50.32 ± 1.48^{a}	48.14 ± 1.33^{a}	45.26 ± 1.20^{a}	40.36 ± 1.32^{a}	24.78 ± 0.20^{b}	15.28 ± 0.10^{b}	08.32 ± 0.00^{b}
P3 (RFE plus 15% EY plus 1.25 mM GSH)	60.41 ± 1.31^{a}	50.12 ± 1.27^{a}	43.11 ± 1.24^{a}	40.61 ± 1.16^{a}	38.17 ± 1.17^{a}	30.28 ± 0.10^{c}	21.19 ± 0.08^{c}	09.51± 0.04^{c}	06.10± 0.00^{b}

P: Treatment, D: Storage days, ^{abcd} Means with different superscripts within the same column indicate highly significant differences ($p < 0.01$), (P0): Red fruit extract extender plus 15% egg yolk, (P1): Red fruit extract extender plus 15% egg yolk plus 0.75 mM glutathione, (P2): Red fruit extract extender plus 15% egg yolk plus 1 mM glutathione, (P3): Red fruit extract extender plus 15% egg yolk plus 1.25 mM glutathione.

The current results were similar to those of Agung et al. (2023), in which the addition of 0.75 mM and 1 mM glutathione resulted in higher responses in Limousin cattle. Fitri et al. (2025) indicated that adding glutathione at a concentration of 1 mM notably increased the progressive motility of spermatozoa during semen storage at 4°C. In comparison to the study conducted by Carriço et al. (2023), administering 1 mM glutathione exhibited a more considerable reduction in oxidative stress than 0.75 mM, suggesting potential advantages for improving liquid semen quality over a longer period. The differences in these results were likely attributable to several factors, including different cattle breeds that may influence physiological responses to glutathione supplementation, the composition of the semen extender, which affects membrane stability and enhances antioxidant efficacy, and semen storage conditions that can impact spermatozoa metabolism (Ansari et al., 2021; Agung et al., 2023). Red fruit extract functions as a natural source of antioxidants, egg yolk serves as both a physical protector and a nutrient provider that maintains spermatozoa membrane stability, while glutathione enhances the internal antioxidant defense system of spermatozoa and preserves mitochondrial function to support motility and viability (Ervandi et al., 2023; Chen et al., 2024). Carriço et al. (2023) reported that glutathione supplementation in diluents functions as an antioxidant, protecting cells from oxidative damage and supporting detoxification. Ogata et al. (2022) reported that adding glutathione reduces the level of lipid peroxidation and helps maintain the stability of semen membranes during storage of liquid semen at 4°C. This plays an important role in maintaining the morphological integrity of semen, especially during long-term liquid storage. Solihati et al. (2018) and Zou et al. (2021) suggested that the use of glutathione in dilution media is expected to reduce the emergence of free radicals that can damage the plasma membrane. Shah et al. (2017) indicated that glutathione helps sustain the survival of cattle spermatozoa by inhibiting apoptosis and cryocapitation pathways, thereby protecting spermatozoa from the adverse effects of cryopreservation on semen. In addition, Khan et al. (2021) demonstrated that glutathione supplementation in liquid bovine semen considerably reduced DNA fragmentation. Biological variations among cattle breeds, such as sperm cell size, enzyme levels, and sensitivity to oxidative stress and temperature changes, also affect the spermatozoa's ability to survive during storage. Berry et al. (2019) found that genetic variation among cattle breeds contributes to significant differences in semen quality, including volume and spermatozoa viability. Krishna et al. (2024) reported that antioxidant enzyme activities, such as superoxide dismutase and catalase, play a crucial role in maintaining semen quality; decreased activity of these enzymes leads to increased oxidative damage to spermatozoa.

The current results indicated that motility persisted until the five days of semen storage with P0 treatment, followed by P3 treatment. This was likely due to the toxic effects of excessive antioxidant accumulation in liquid semen storage media. The findings of Agung et al. (2023) indicated that glutathione concentrations higher than 1 mM may disrupt the spermatozoa reduction system, leading to decreased spermatozoa motility and viability in cattle. This imbalance can indirectly cause an increase in reactive oxygen species production and exacerbate spermatozoa damage. Carriço et al. (2023) reported that supplementation with glutathione at concentrations exceeding 2 mM results in a progressive decline in sperm motility, accompanied by increased spermatozoa mortality in cattle. The administration of glutathione at high concentrations has been demonstrated to induce morphological abnormalities in the head and tail regions of bovine spermatozoa (Zou et al., 2021). This effect is associated with alterations in the plasma membrane of spermatozoa resulting from an imbalance of glutathione in the semen storage environment. Semen quality supplemented with glutathione at concentrations above 1.5 mM exhibited reduced ability to penetrate oocytes, resulting in lower fertilization success rates in AI programs in cattle (Triwulaninngsih et al., 2003).

Sperm membrane integrity

The mean membrane integrity of PO bulls' spermatozoa after storage, measured on the first day and across six days at 50°C, is shown in Table 3. The present results indicated that the treatment of glutathione level supplementation in liquid semen diluent had a very significant effect ($p < 0.01$) on the percentage of membrane integrity of PO bulls' spermatozoa after six days of cold storage. The highest membrane integrity was observed in the P1 and P2 diluent treatments, with average values of 68.10 ± 0.02% and 60.12 ± 0.04%, respectively, compared to the P0 and P3 treatments. Additionally, the current results demonstrated a very significant difference ($p < 0.01$) in the membrane integrity of PO bulls on the sixth day of semen storage. Duncan's post hoc test demonstrated that the P1 and P2 diluent treatments produced the best results after six days of storage.

Table 3. Mean of sperm membrane integrity after preservation at 5°C

Treatment	Mean spermatozoa membrane integrity (%) ± Standard deviation		
	Fresh	D-1	D-6
P0 (RFE plus 15% EY)	88.16 ± 1.40^{a}	70.18 ± 1.13^{c}	49.16 ± 0.06^{c}
P1 (RFE plus 15% EY plus 0.75 mM GSH)	88.16 ± 1.40^{a}	85.02 ± 0.06^{a}	68.10 ± 0.02^{a}
P2 (RFE plus 15% EY plus 1 mM GSH)	88.16 ± 1.40^{a}	80.12 ± 1.08^{b}	60.12 ± 0.04^{b}
P3 (RFE plus 15% EY plus 1.25 mM GSH)	88.16 ± 1.40^{a}	63.13 ± 1.17^{d}	40.10 ± 0.07^{d}

P: Treatment, D: Storage days, ^{abcd} Means with different superscripts within the same column indicate highly significant differences ($p < 0.01$), (P0): Red fruit extract extender plus 15% egg yolk, (P1): Red fruit extract extender plus 15% egg yolk plus 0.75 mM glutathione, (P2): Red fruit extract extender plus 15% egg yolk plus 1 mM glutathione, (P3): Red fruit extract extender plus 15% egg yolk plus 1.25 mM glutathione.

The present results were similar to those of Fitri et al. (2025), who found that adding 0.75 mM glutathione to the cattle semen freezing medium had a significant positive effect on semen quality parameters, including motility, cell membrane integrity, and a decrease in the level of oxidative damage to spermatozoa. A dose of 0.75 mM can offer optimal protection for semen quality during cryopreservation (Zou et al., 2021), likely attributable to the glutathione antioxidant properties, which can mitigate or prevent damage to the sperm plasma membrane caused by free radicals. The combination of red fruit extract, abundant in natural antioxidants such as β-carotene and tocopherol, effectively scavenges free radicals. Egg yolk acts as a membrane protector owing to its rich phospholipid and lipoprotein composition, while glutathione plays a pivotal role in maintaining intracellular redox homeostasis and enhancing the endogenous antioxidant defense system (Solihati et al., 2018; Ervandi et al., 2023; Chen et al., 2024). The addition of 0.75 mM glutathione has been shown to reduce lipid peroxidation, preserve membrane integrity, and enhance the motility of bovine spermatozoa following storage (Ducha et al., 2024). A glutathione dose of 0.75 mM is generally considered optimal, as it provides adequate protection for semen without inducing adverse effects (Fitri et al., 2025). Using lower or higher doses might not achieve the expected membrane protection and semen motility improvements after refrigeration. Carriço et al. (2023) demonstrated that supplementation with 1 mM glutathione in bovine semen extenders can enhance sperm membrane integrity.

Red fruit extract contains α-tocopherol, β-carotene, oleic acid, linoleic acid, linolenic acid, vitamin C, calcium, phosphorus, and iron (Tethool et al., 2021). A formulation of red fruit extract with 15% egg yolk and 0.75 mM glutathione could maintain membrane integrity in PO bull's spermatozoa during six days of storage. This was likely attributable to the red fruit containing tocopherol and β-carotene, which protected spermatozoa cells from morphological damage, while glutathione functioned as an antioxidant capable of protecting the membrane of PO bull's spermatozoa. A combination of antioxidants, namely tocopherol and β-carotene, can complement each other in maintaining the oxidative status of spermatozoa. Tocopherol and β-carotene functioned as external antioxidants from the extender, whereas glutathione served as an internal antioxidant in the sperm cells. This combination of antioxidants could improve post-

thaw semen quality, including sperm motility and structural integrity, as well as enhance *in vitro* fertilization outcomes (Luo et al., 2022). Solihati et al. (2018) and Zou et al. (2021) applied glutathione in the dilution medium, which was expected to reduce the emergence of free radicals that could damage the plasma membrane. Antioxidants, such as glutathione, which can reduce oxidative damage, are crucial in preserving bovine semen (Fitri et al., 2025).

Sperm acrosome status

The average percentage of PO bulls' sperm acrosome status after storage for one to six days at a temperature of 5°C is shown in Table 4. The current results indicated that supplementing glutathione levels in semen diluent had a highly significant effect ($p < 0.01$) on the percentage of non-capacity of PO bulls' spermatozoa after six days of cold storage. The highest non-capacity was observed in the P1 and P2 treatments, with average values of 73.11 ± 0.05% and 65.19 ± 0.09%, respectively, compared to the P0 and P3 treatments. Additionally, the current results indicated a very significant difference ($p < 0.01$) in the non-capacity of PO bulls until the sixth day of semen storage. Duncan's post hoc test demonstrated the highest results for the P1 and P2 treatments after six days of storage.

Table 4. Mean of non-capacity after preservation at 5°C

Treatment	Mean of non-capacity (%) ± Standard deviation		
	Fresh	D-1	D-6
P0 (RFE plus 15% EY)	82.24 ± 1.30^{a}	70.30 ± 1.13^{c}	57.16 ± 0.15^{c}
P1 (RFE plus 15% EY plus 0.75 mM GSH)	82.24 ± 1.30^{a}	83.13 ± 1.03^{a}	73.11 ± 0.05^{a}
P2 (RFE plus 15% EY plus 1 mM GSH)	82.24 ± 1.30^{a}	73.20 ± 1.08^{b}	65.19 ± 0.09^{b}
P3 (RFE plus 15% EY plus 1.25 mM GSH)	82.24 ± 1.30^{a}	67.12 ± 1.20^{d}	51.20 ± 0.14^{d}

P: Treatment, D: Storage days, ^{abcd} Means with different superscripts within the same column indicate highly significant differences ($p < 0.01$), (P0): Red fruit extract extender plus 15% egg yolk, (P1): Red fruit extract extender plus 15% egg yolk plus 0.75 mM glutathione, (P2): Red fruit extract extender plus 15% egg yolk plus 1 mM glutathione, (P3): Red fruit extract extender plus 15% egg yolk plus 1.25 mM glutathione.

The current findings were similar to those of Fitri et al. (2025), who indicated that supplementing cattle semen freezing medium with 0.75 mM glutathione significantly improved semen quality, enhancing motility, cell membrane integrity, and reducing oxidative damage to spermatozoa. A dose of 0.75 mM provided optimal protection for semen quality during cryopreservation, with the best results observed for the P1 and P2 treatments, maintaining above 60% viability on the sixth day (Zou et al., 2021). These findings were likely due to the relatively high capacitation of PO bulls' spermatozoa, indicating that the spermatozoa had intact membranes, indicating that calcium ions were evenly distributed in the spermatozoa head. This condition was influenced by membrane cholesterol, indicating that the semen used in the present study was suitable for use in AI procedures in the field (Ervandi et al., 2020). Gualtieri et al. (2010) noted that reducing spermatozoa membrane cholesterol leads to increased permeability to calcium and bicarbonate, thereby helping to decrease spermatozoa that have not undergone capacitation. The contents of tocopherol, β-carotene, and ascorbic acid in red fruit extract, which also contains glucose, protein, fat, vitamin C, antioxidants, and glutathione, can strengthen the internal antioxidant defense system of spermatozoa and preserve mitochondrial function, thereby protecting the morphology of PO bulls' spermatozoa from damage (Suyadi et al., 2020; Ervandi et al., 2023). The content of red fruit, such as tocopherol, carotenoids, and β-carotene, can influence abnormalities that occur in spermatozoa (Tethool et al., 2021). The optimal dose of 0.75 mM glutathione improved sperm capacitation and fertilizing ability by enhancing motility and acrosome function (Fitri et al., 2025). Glutathione supplementation can help reduce oxidative damage to spermatozoa stored at low temperatures, such as 5°C, and improve semen quality after thawing (Izanloo et al., 2021).

The current findings indicated that the red fruit extract formulation with 15% egg yolk and 0.75 mM glutathione had a very significant effect ($p < 0.01$) on the percentage of PO bulls' spermatozoa capacitation after six days of cold storage, as shown in Table 5.

Table 5. Mean of capacitance after preservation at 5°C

Treatment	Mean of non-capacity (%) ± Standard deviation		
	Fresh	D-1	D-6
P0 (RFE plus 15% EY)	10.06 ± 1.31^{a}	11.52 ± 1.40^{c}	36.13 ± 0.23^{c}
P1 (RFE plus 15% EY plus 0.75 mM GSH)	10.06 ± 1.31^{a}	11.09 ± 1.10^{a}	13.10 ± 0.09^{a}
P2 (RFE plus 15% EY plus 1 mM GSH)	10.06 ± 1.31^{a}	11.17 ± 1.33^{b}	21.62 ± 0.10^{b}
P3 (RFE plus 15% EY plus 1.25 mM GSH)	10.06 ± 1.31^{a}	11.62 ± 1.52^{d}	55.10 ± 0.12^{d}

P: Treatment, D: Storage days, ^{abcd} Means with different superscripts within the same column indicate highly significant differences ($p < 0.01$), (P0): Red fruit extract extender lus 15% egg yolk, (P1): Red fruit extract extender plus 15% egg yolk plus 0.75 mM glutathione, (P2): Red fruit extract extender plus 15% egg yolk plus 1 mM glutathione, (P3): Red fruit extract extender plus 15% egg yolk plus 1.25 mM glutathione.

The Least Duncan's post hoc test indicated the best results for the P1 and P2 diluent treatments after a storage period of 6 days. The present results were similar to those of Fitri et al. (2025), who found that adding 0.75 mM glutathione to the cattle semen freezing medium had a significant positive effect on semen quality parameters, including motility, cell membrane integrity, and a decrease in oxidative damage to spermatozoa. A dose of 0.75 mM can provide optimal protection for semen quality during cryopreservation (Zou et al., 2021). Obtained the best observation results of P1 diluent treatment with a storage period of 6 days below 20%. The present results indicated that the red fruit extract with 15% egg yolk plus 0.75 mM glutathione (P1) was significantly different from the P0, P2, and P3 treatments. Consistent with the findings of Fitri et al. (2025), glutathione at 0.75 mM was shown to effectively protect spermatozoa from oxidative stress and enhance capacitation efficiency. Red fruit extract acts as a natural protector due to its antioxidant content, egg yolk supplies energy and helps maintain membrane stability in spermatozoa, and glutathione protects spermatozoa from oxidative stress (Ervandi et al., 2023). Zou et al. (2021) reported that glutathione at a concentration of 0.75 mM is effective in enhancing the quality of bovine semen during capacitation and in preventing oxidative damage to the sperm membrane. It was found that glutathione at a concentration of 0.75 mM can improve the quality parameters of bovine spermatozoa, including motility, viability, and membrane integrity (Fitri et al., 2025). Capacitation can be triggered by various factors, including changes in pH, calcium ion levels, and the formation of reactive oxygen species (Tethool et al., 2021). During the capacitation process, changes occur in the spermatozoon's plasma membrane, such as a decrease in plasma membrane proteins and changes in ionic permeability that enable the spermatozoon to undergo the acrosome reaction necessary to penetrate the ovum (Beltrán et al., 2016). Some of the key changes that occur during capacitation include alternation in spermatozoa membrane proteins, increased ionic permeability, and enzyme activation that enables the acrosome reaction to occur (Hitit et al., 2020).

The present findings demonstrated that the application of red fruit extract combined with 15% egg yolk and 0.75 mM glutathione had a very significant effect ($p < 0.01$) on the acrosome reaction variable in PO bulls during storage days one to six. The Least Duncan's post hoc test revealed that P1 treatment was significantly different from the P1, P2, and P3 treatments. This finding was probably attributable to the low percentage of acrosome reactions observed in each diluent treatment, suggesting that there was no significant spermatozoa damage exceeding 50%, which could adversely affect the fertility potential of PO bulls' semen. Hitit et al. (2020) indicated that spermatozoa lose their fertility when the acrosome is damaged by more than 50%. According to Muhammad et al. (2019), damage to the acrosomal membrane impairs its protective role, which is critical for spermatozoa function. The observed differences among treatments were likely due to their influence on the sperm plasma membrane, which protected the underlying acrosome. The integrity of this membrane in PO bull spermatozoa is highly dependent on its cholesterol content, which the treatments appeared to have modulated. The high cholesterol content in the membrane makes it more flexible, and when cholesterol is low in the membrane, it causes spermatozoa to be easily damaged (Muhammad et al., 2019). Adding glutathione at a concentration of 0.75 mM significantly improves spermatozoa quality, including accelerating the acrosome reaction (Ducha et al., 2024). Added glutathione to semen thinners plays an important role in maintaining the stability of spermatozoa membranes and improving acrosome function (Wadhwa et al., 2024). Glutathione can affect the acrosome reaction in cattle by increasing the ability of spermatozoa to penetrate the zona pellucida of oocytes (Ahmad et al., 2021).

CONCLUSION

Red fruit extract with 15% egg yolk and 0.75 mM glutathione diluent demonstrated superior results compared to other diluent treatments. These findings were indicated by spermatozoa that maintained membrane integrity and had not undergone capacitation, remaining elevated, while spermatozoa that had undergone capacitation and acrosome reaction remained low. The diluent formulation of red fruit extract with 15% egg yolk and 0.75 mM glutathione was able to maintain acrosome status above 50% after six days of cold storage. Therefore, the combination of red fruit extract, 15% egg yolk, and 0.75 mM glutathione was identified as the optimal formulation for liquid semen preservation, meeting the required standards for practical application in AI programs in the field. Furthermore, future studies should be conducted on fertility assessments related to semen quality utilizing AI technology.

DECLARATIONS

Authors' contributions

Mohamad Ervandi wrote the manuscript, designed the study, supervised the study, and reviewed the final version of the manuscript. Talha Dangkua collected and analyzed the data. All authors have read and approved the final edition of the manuscript.

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Ethical considerations

All authors have been screened for ethical issues, including plagiarism, consent for publication, ethical violations, data falsification, and multiple publications or submissions. AI was not used to generate data, perform statistical analyses, or interpret the research findings. All sections of the manuscript related to the methodology, results of the study, and conclusions were written entirely by the author based on the findings of the present study.

Availability of data and materials

The authors confirm that all data supporting the findings of this study are included within the manuscript.

Conflict of interests

The authors declared no conflicts of interest.

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