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Effects of Adding Glutathione to CEP Diluent on Post-Thawing Sperm Quality of Bali Cattle

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Abstract
Storing spermatozoa at freezing temperatures can expose spermatozoa cells to reactive oxygen species (ROS) and damage their quality. Therefore, the spermatozoa require storage in certain diluents treated with antioxidants. This study aims to determine the effect of adding glutathione as an antioxidant in CEP diluent on the quality of frozen Bali cattle semen. This study used a Completely Randomized Design with six doses of glutathione (0, 0.5, 0.75, 1, 1.25, and 1.5 mM) in CEP diluent with four repetitions. Fresh semen obtained from Balinese cows is approximately six years old and collected using the Artificial Vagina method. The ANOVA test showed that adding various doses of glutathione significantly affected the quality of post-thawing spermatozoa ($P<0.05$). Duncan's test showed that 0.75 mM glutathione had the highest post-thawing for all parameters, motility (55.30 ± 2.95%), viability (71.26 ± 2.23%), and membrane integrity (58.08±2.52%). This study concluded that adding 0.75 mM glutathione was the best dose to maintain the quality of Bali cattle spermatozoa post-thawing.

Keywords: antioxidant; freezing semen; genetic diversity and farmed animals; sperm quality
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INTRODUCTION

Indonesia still imports beef because the increase has yet to match beef demand in meat production. The Indonesian Ministry of Agriculture (MOA) targets a Beef Self-Sufficiency Program (*Program Swasembada Daging Sapi*) in 2026 with a total beef cattle population of ±37 million heads. The total beef cattle population from 2020 to 2022 increased from 17.4 million to 17.6 million heads (Badan Pusat Statistik, 2022). However, there are still concerns that the program will not achieve because the total beef cattle population has not yet reached the set number. The problem can be solved by increasing the population of beef cattle that provide meat sources, such as Bali Cattle. The advantage of Bali Cattle is that it has a high carcass percentage in the range of 45 - 55%, so the economic value of Bali Cattle meat is high (Nindhia et al., 2021).

Improvement in the population of Bali cattle can be optimized through the Artificial Insemination (AI) program. One of the success factors of IB is the quality of the semen used. The quality of fresh semen decreases when stored at room temperature because the metabolic process takes place optimally. The energy required by sperm is increasing, but the availability of energy sources is decreasing (Syafi'i and Rosadi, 2022). Efforts to maintain semen quality so that the AI process shows good results can be made by storing semen at freezing temperatures or -196°C (Susilawati et al., 2020).

Spermatozoa cells cannot avoid exposure to free radicals during the freezing process. Silvestre et al. (2021) explain that freezing semen can increase the production of Reactive Oxygen Species (ROS). Ducha (2018) added that free radicals trigger lipid peroxidation, which affects spermatozoa's motility, metabolism, structure, and fertility. Reactive Oxygen Species (ROS) production impacts damage to spermatozoa lipid membranes, resulting in decreased viability (Zhang et al., 2021). Therefore, storing semen at low temperatures requires the addition of diluents so that the quality of spermatozoa is maintained (Ducha et al., 2020).

One of the bovine semen diluents is Caudal Epididymal Plasma (CEP) diluent. CEP diluent is developed with an ionic composition, pH, and osmolarity, almost resembling bovine epididymis. It was

first developed by Verberckmoes et al. (2004) and modified by Ducha (2012) using different antibiotics, egg yolk concentrations, and methods. CEP diluent consists of NaCl, KCl, $CaCl_2(H_2O)$, $MgCl_2(H_2O_6)$, $NaHCO_3$, NaH_2PO_4, KH_2PO_4, fructose, sorbitol, Tris, BSA, citric acid, streptomycin, penicillin and 20% egg yolk (Ducha, 2018).

It has been previously explained that freezing cow semen can produce ROS. Mammalian semen contains enzymatic antioxidants which can be reduced if the amount of ROS is excessive (Silvestre et al., 2021). One example of an enzymatic antioxidant is glutathione peroxidase (GPx) and glutathione reductase (GR). These two enzymes interact to remove hydrogen peroxide (H_2O_2) to form glutathione oxidase (GSSG) and water (H_2O). Glutathione reductase (GR) recycles GSSG by adding NADPH to become reduced glutathione (GSH) (Adeoye et al., 2018). According to Kankofer et al. (2005), a combination of exogenous and endogenous antioxidants can reduce excess ROS.

Efforts to overcome the presence of ROS can be made by adding antioxidant substances in bovine semen diluent. Ducha et al. (2023) stated that adding antioxidants to the diluent is needed to minimize the occurrence of lipid peroxidation. Nonenzymatic antioxidants such as glutathione can be added to the diluent semen. Glutathione is a tripeptide compound formed of glutamic acid, cysteine, and glycine (Kalinina et al., 2014). Recent research by (Bebas et al., 2023) showed that the addition of 1 mM in Andromed diluent can maintain the frozen sperm quality of Bali Cattle where the motility is 46.75%, intact acrosomal membrane is 55.75%, and intact plasma membrane is 63.75%.

This study is a development in using CEP diluent by adding various doses of glutathione to the quality of Bali Cow spermatozoa post-thawing. The novel aspect of this study is the addition of nonenzymatic antioxidant glutathione in CEP diluent. This study aimed to determine the effect of adding the best dose of glutathione in CEP diluent on the sperm motility, viability, and integrity membrane of Bali Cattle.

MATERIALS AND METHODS

This study was experimental using a Completely Randomized Design (CRD). This study consisted of 6 treatments of glutathione doses in CEP diluent with four repetitions: P0 (control); P1 (0.5 mM glutathione); P2 (0.75 mM glutathione); P3 (1 mM glutathione); P4 (1.25 mM glutathione); and P5 (1.5 mM glutathione).

CEP diluent material refers to Ducha (2018), the composition of one liter consists of 15 mmol NaCl; 7.0 mmol KCl; 3.0 mmol $CaCl_2(H_2O)_2$; 3.0 mmol $NaHCO_3$; 3.0 mmol $MgCl_2$ $(H_2O)_6$; 11.9 mmol $NaHCO_3$; 8.0 mmol NaH_2PO_4; 20.0 mmol KH_2PO_4; 55 mmol fructose; 1.0 g sorbitol; 2.0 g BSA; 133.7 mmol Tris; 1000 IUI penicillin; 1 g streptomycin; and 42.6 mmol citric acid. All ingredients were made in aliquots using Otsuka sterile water for injection. The solution is divided into six parts to add various doses of glutathione. Next, the solution was sterilized using a 0.22 µm millipore membrane, supplemented with 20% egg yolk, and homogenized. The solution was deposited for approximately three days in the refrigerator until it formed two layers, namely the supernatant and the precipitate. The supernatant was separated using a syringe and used as a diluent.

Fresh semen was collected from Bali cattle at the Artificial Insemination Center (BBIB) Singosari, Malang, by the Artificial Vagina (AV) method. Macroscopic evaluation of fresh semen included volume, color, pH, odor, and consistency. Microscopic evaluation included concentration, mass motility, individual motility, abnormality, viability, and spermatozoa membrane integrity.

The spermatozoa dilution process began with A1 and A2 dilutions at 37℃ room temperature (Rahayu and Ducha, 2022). In the A1 dilution, the amount of diluent volume added is proportional to the volume of fresh semen (1:1). Next, dilution A2 by adding total diluent according to the following formula:

$$V.\,\text{total} = \frac{\text{V. semen x sperm concentration } (10^6)}{(\frac{25 \text{ x } 10^6}{0.25})}$$

$$\text{V. A2} = \text{V. total} - (\text{V. semen} + \text{V. A1} + \text{V. B})$$

The liquid semen (semen + dilution A) was stored in the refrigerator until the temperature reached 5℃. Next, dilution B (diluent A and 13% glycerol) was added as much as half of the total volume (Rahayu and Ducha, 2022). Semen freezing began with the post-freezing process, a gradual decrease in temperature from 5℃ to -140℃ for 7 minutes using a DigitCool machine. Furthermore, the freezing process used liquid nitrogen at -196℃.

Spermatozoa motility was evaluated objectively by Computer-Assisted Sperm Analysis (CASA) (37°C) by dripping semen on an object glass covered by a cover glass. The term for a good spermatozoa motility percentage is ≥ 40% (SNI, 2021). Spermatozoa with good motility move progressively and form waves (Tanii et al., 2022).

Spermatozoa viability was evaluated subjectively by dripping semen on object glass and giving a drop of eosin negrosin dye (1:2), then making a thin smear. The observation was carried out under a microscope at 400x magnification (Utami and Ducha, 2023), and 200 spermatozoa were assessed per sample. The term for the percentage of spermatozoa viability is at least 50% (Ducha, 2018). The percentage of spermatozoa viability uses the following formula:

$$\%\text{Viability} = \frac{\text{total of live sperm}}{\text{total sperm (live and dead)}} \text{ x } 100\%$$

Spermatozoa plasma membrane integrity was evaluated using a Hypo Osmotic Swelling Test (HOST) solution consisting of 1.3 g of fructose and 0.7 trisodium citrate dehydrate in 100 ml of distilled water. Semen samples were dissolved into the HOST solution (ratio 1: 10), incubated at 37℃ for 30 minutes, dripped on an object glass, and made a thin smear. The preparations were observed with a microscope at 400x magnification, and 200 spermatozoa were assessed per sample. Spermatozoa having a coiled tail indicates that the plasma membrane is still intact, while a straight tail suggests that the spermatozoa plasma membrane has been damaged (Mujahidurrohman et al., 2023). The percentage of integrity that is suitable for artificial insemination is at least 50%. Percentage of spermatozoa membrane integrity using the following formula:

$$\%\text{Membran Integrity} = \frac{\text{total of coiled tail sperm}}{\text{total sperm}} \text{ x } 100\%$$

The results were tested for normality and homogeneity to determine the distribution of the data. Normally distributed and homogeneous data continued ANOVA test. Data showed significance ($P < 0.05$), and then Duncan's test was to determine the fundamental differences between treatments. The entire data analysis process used SPSS 25 for Windows.

RESULTS

Fresh semen quality of Balinese cattle aged ±6 years was evaluated macroscopically and microscopically. Fresh semen was included in the normal category because it has motility ≥70% and abnormality ≤20%. These results indicated that the fresh semen was of good quality (SNI, 2021). The results of the evaluation are presented in Table 1.

Table 1. Results of observation of the quality of fresh semen of Balinese cattle

Parameters	Average ± Standard Deviation
Color	White milk
pH	6.52 ± 0.10
Consistency	Thin creamy
Volume (ml)	7.85 ± 1.4
Odor	Specific
Concentration Sperm (juta/ml)	982 ± 234
Mass Motility	++
Progressive motility (%)	72.2 ± 4.17
Abnormality (%)	7.13 ± 1.13
Viability (%)	85.78 ± 9.30
Membrane Integrity (%)	65.71± 8.34

The results of adding glutathione in CEP diluent on sperm motility before freezing and post-thawing were presented in Table 2. Motility before freezing was highest at a dose of 0.75 mM (70.90±1.43%) and lowest at a dose of 1.5 mM (64.9±0.61%). Motility post-thawing was highest at 0.75 mM (55.30±2.95%) and lowest at a dose of 1.5 mM (41.73±0.94%). The sperm motility before freezing and post-thawing showed an increase in 0.75 mM glutathione and a decrease with the high dose of glutathione.

The ANOVA test showed sig. $P<0.05$ meant adding various doses of glutathione in CEP diluent affects the sperm motility of before-freezing and post-thawing spermatozoa. The Duncan test showed 0.75 mM glutathione had the best average percentage of motility before freezing (70.90±1.43%) and post-thawing (55.30±2.95%) compared to other treatments.

Table 2. The average ± SD of sperm motility in Balinese cattle with the addition of various doses of glutathione in CEP diluent

Doses (mM)	Average Motility Percentage (%) ± Standard Deviation		Reduction (%)
	Before Freezing	Post-Thawing	
0	68.50 ± 1.39 b	52.85 ± 1.47 ab	15.7
0.5	66.96 ± 0.60 b	49.89 ± 1.22 b	17.5
0.75	70.90 ± 1.43 a	55.30 ± 2.95 a	15.6
1	67.26 ± 1.56 b	52.05 ± 2.32 b	15.2
1.25	67.05 ± 1.10 b	44.06 ± 2.16 c	23.0
1.5	64.60 ± 0.61 c	41.73 ± 0.94 c b	22.9

Remarks: The difference in notation (a, b, c) showed a fundamental difference between treatments (P<0.05). Notation a: best percentage notation; C notation: Lowest percentage notation

The results of the addition of glutathione in CEP diluent on sperm viability before freezing and post-thawing were presented in Table 3. Viability before freezing was highest at a dose of 0.75 mM (84.04±2.52%) and the lowest at a dose of 1.5 mM (74.9±1.10%). Viability post-thawing was highest at 0.75 mM (71.26±2.23%) and the lowest at a dose of 1.5 mM (65.25±1.57%). Sperm viability before freezing and post-thawing showed an increase in the addition of 0.75 mM and a decrease with the high dose of glutathione.

The ANOVA test showed that adding glutathione in CEP diluent affects sperm viability before freezing and post-thawing (P < 0.05). The Duncan test showed that 0.75 mM glutathione had the best average viability percentage before freezing (84.04±2.52%) and post-thawing (71.26±2.23%) compared to other treatments.

Table 3. The average ± SD of sperm viability in Balinese cattle with the addition of various doses of glutathione in CEP diluent

Doses (mM)	Average Viability Percentage (%) ± Standard Deviation		Reduction (%)
	Before Freezing	Post-Thawing	
0	77.81 ± 2.10 b	69.85 ± 1.83 ab	8.0
0.5	76.6 ± 3.7 b	67.54 ± 2.57 bc	9.1
0.75	84.04 ± 2.52 a	71.26 ± 2.23 a	12.8
1	77.65 ± 2.86 b	69.24 ± 1.39 ab	8.4
1.25	76.51 ± 3.29 b	67.08 ± 1.85 bc	9.4
1.5	74.90 ± 1.10 b	65.25 ± 1.57 c	9.7

Remarks: The difference in notation (a, b, c) showed a fundamental difference between treatments (P<0.05). Notation a: best percentage notation; C notation: Lowest percentage notation

The results of sperm viability using eosin-negrosin staining can be seen in Figure 1. Results show that the 0.75 mM glutathione has the highest number of live spermatozoa. Live spermatozoa are indicated by clear or unstained heads, while dead spermatozoa have purplish redheads.

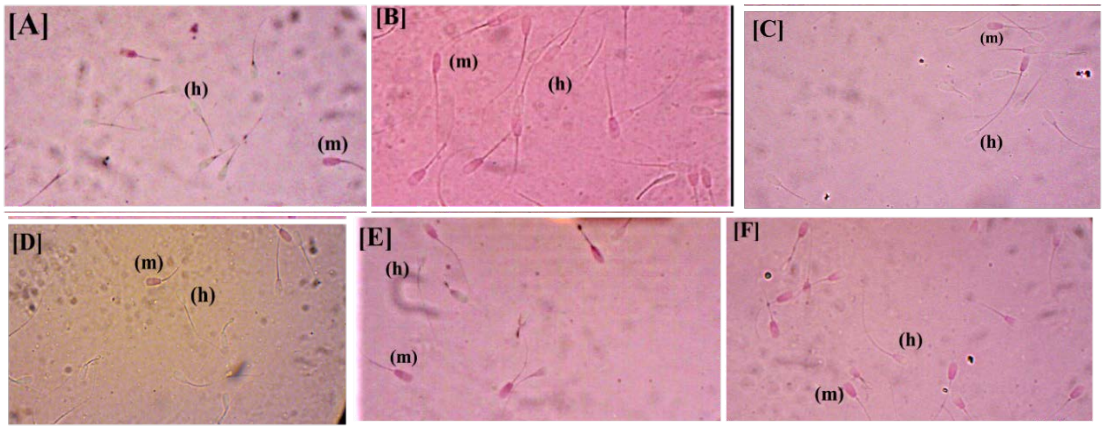

Figure 1. Sperm viability of Balinese cattle post-thawing with 400x magnification. Description: [A] 0 mM; [B] 0.5 mm; [C] 0.75 mm; [D] 1 mM; [E] 1.5 mM; [F] 1.75 mm; (h) living spermatozoa; and (m) dead spermatozoa.

The results of adding glutathione in CEP diluent on sperm plasma membrane integrity before freezing and post-thawing were presented in Table 4. The highest percentage of membrane integrity

before freezing was known at a dose of 0.75 mM at 65.55 ± 4.59% and the lowest at 1.75 mM at 56.21 ± 3.55%. The integrity of the sperm plasma membrane before freezing was highest at 0.75 mM (58.08± 2.52%) and lowest at 1.75 mM (49.29±5.03%). The sperm plasma membrane integrity before freezing and post-thawing showed an increase in the addition of 0.75 mM and a decrease with the higher dose of glutathione. Based on adding glutathione in CEP showed a decrease along with the higher dose of glutathione added.

The ANOVA test showed that adding glutathione in the CEP diluent affects the integrity of the sperm membrane before freezing and post-thawing (P<0.05). The Duncan test showed that adding 0.75 mM glutathione had the best percentage of integrity before freezing (65.55 ± 4.59%) and post-thawing (58.08 ± 2.52%) compared to other treatments.

Table 4. The average ± SD of the sperm plasma membrane integrity in Balinese cattle with the addition of various doses of glutathione in CEP diluent

Doses (mM)	Average membrane integrity (%) ± Standard Deviation		Reduction (%)
	Before Freezing	Post-Thawing	
0	63.92 ± 3.56 ^{ab}	56.44 ± 2.98 ^{ab}	7.5
0.5	60.68 ±1.77 ^{abc}	51.45 ± 4.64 ^{bc}	9.2
0.75	65.55 ± 4.59 ^{a}	58.08 ± 2.52 ^{a}	7.5
1	61.06 ± 4.36 ^{abc}	53.81 ± 0.72 ^{abc}	7.3
1.5	58.84 ± 3.51 ^{bc}	51.20 ± 1.98 ^{bc}	7.6
1.75	56.21 ± 3.55 ^{c}	49.29 ± 5.03 ^{c}	6.9

Remarks: The difference in notation ($^{a, b, c}$) showed a fundamental difference between treatments (P<0.05). Notation ^{a}: best percentage notation; ^{C} notation: Lowest percentage notation

The results of the integrity of sperm membrane preparations that have been incubated in HOST solution can be seen in Figure 2. The results of 0.75 mM glutathione treatment showed that spermatozoa have more intact membranes than other treatments. Coiled-tail spermatozoa indicate an intact membrane, while straight-tail spermatozoa indicate intact membrane.

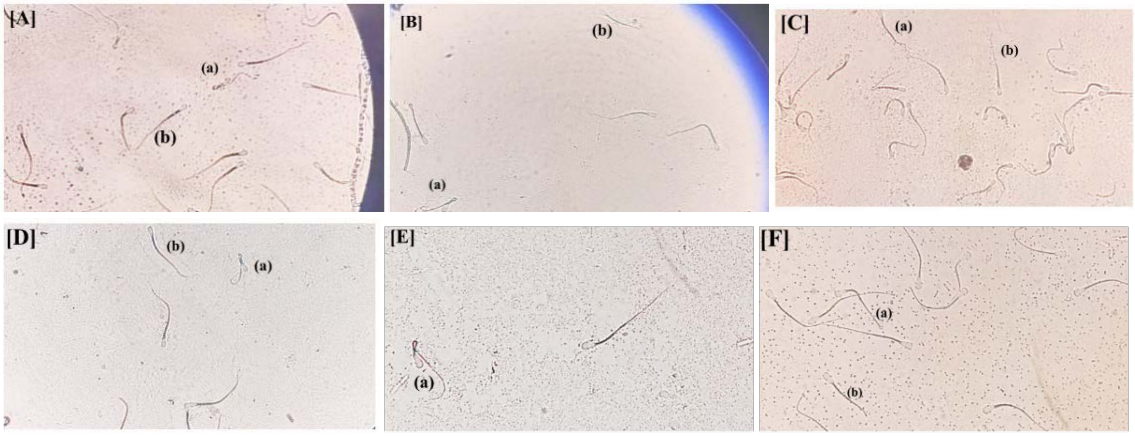

Figure 2. The sperm plasma membrane integrity in Balinese cattle post-thawing with 400x magnification. Description: [A] 0 mM; [B] 0.5 mm; [C] 0.75 mm; [D] 1 mM; [E] 1.5 mM; [F] 1.75 mm; (a) coil-tailed spermatozoa; and (b) straight-tailed spermatozoa.

DISCUSSION

Fresh semen of 6-year-old Balinese cattle was evaluated macroscopically and showed the average volume was ±7.85 ml; it has a characteristic odor, milky white; pH 6.5; and a thin creamy consistency. Whereas the microscopic evaluation showed that the sperm concentration was 982 million/ml; mass motility (2+), progressive motility was 72.2%; abnormality was 7.1%, viability was 85.78%, and integrity of sperm membrane was 65.71% (Table 1). The results showed that progressive motility ≥70% and abnormality ≤20% are suitable according to SNI (2021), so can dilute to media diluent.

The average volume of ejaculated by Bali Cattle is included in the normal category. It is higher than the results of Blegur et al. (2020), which is 4 ml. Different cattle ages cause the difference in volume. Setiawan et al. (2020) explained that Balinese cattle have an increasing volume of semen at 4-6 years old due to the growth and development of reproductive organs. The average sperm concentration was 982 million/ml in the watery to moderate category. The collection frequency can influence the difference in

sperm concentration between individuals (Komariah et al., 2020). Progressive motility is movement in sperm cells and an essential parameter for determining male fertility (Manehat et al., 2021). Various factors that affect sperm motility are male age, male nation, genetics, ejaculation frequency, feed, and environment (Kafiar et al., 2019).

Efforts to optimize the Artificial Insemination (IB) program can be made by maintaining the quality of spermatozoa used, so spermatozoa need to be stored properly. This study was conducted by storing spermatozoa cells in CEP diluent and then continuing with the freezing process. The presence of fructose in CEP diluent can act as the main energy source for spermatozoa cells during the cooling process (Bustani and Baiee, 2021). Furthermore, the freezing process at -196 ℃ can cause spermatozoa cells to experience cold shock, resulting in structural and functional damage to spermatozoa. The addition of 13% glycerol in CEP diluent acts as an intracellular cryoprotectant and is added before the freezing process. According to Rozi and Ducha (2021), glycerol can balance water in spermatozoa cells through the mechanism of modifying ice crystals and inhibiting membrane damage.

Bovine spermatozoa cells have enzymatic antioxidants that function to reduce the production of free radicals or ROS, namely glutathione peroxidase (GPx) and glutathione reductase (GR). Adeoye et al. (2018) mentioned that the two enzymes react with each other to eliminate hydrogen peroxide (H_2O_2) to glutathione peroxidase (GSSG) and water (H2O). GSSG is recycled by GR by adding NADPH to become reduced glutathione (GSH). These enzymatic antioxidants can be reduced if the amount of ROS is excessive (Silvestre et al., 2021). On the other hand, freezing spermatozoa can increase the production of ROS so that the presence of enzymatic antioxidants is further reduced. This study was conducted to add non-enzymatic antioxidants in CEP diluent to replace the diminishing enzymatic.

Table 2 shows decreasing motility from the initial motility. Therefore, it can be stated that storage at low temperatures can decrease sperm motility. The decrease in sperm motility is due to the appearance of Reactive Oxygen Species (ROS) during storage. This is reinforced by the statement of Blegur et al. (2020) that the impact of sperm storage was the appearance of ROS, which can damage the plasma membrane structure of spermatozoa cells. This damage occurs because the sperm membrane comprises phospholipids of unsaturated fatty acids highly susceptible to ROS (Rodak and Kratz, 2023).

Adding various doses of glutathione in CEP diluent affected sperm motility in Balinese cattle before freezing and post-thawing ($P<0.05$). The motility graph showed it decreased when glutathione was added in a high dose. Adding 0.75 mM glutathione yielded the highest values in sperm motility before freezing (70.90%) and post-thawing (55.30%). The sperm motility post-thawing or frozen sperm is suitable for IB because its value is more than 40% (SNI, 2021). Therefore, it can be concluded that adding 0.75 mM glutathione in the CEP diluent can minimize ROS production during the spermatozoa storage process, corroborated by the research of Maulana et al. (2016), who stated that adding 0,75 mM glutathione in the diluent was able to maintain the sperm motility of Limousine cattle at room temperature for 24 hours.

Glutathione in CEP diluent acts as an antioxidant to scavenge free radicals. Glutathione has a cysteine residue molecule that produces a reactive group and interacts with residues resulting from the disulfide bonding of proteins. Disulfide bonds can be strong antioxidants because they are reactive against hydroxyl radicals (•OH) so they can protect cells from ROS (Adhikari et al., 2020). Cysteine from glutathione acts as an electron donor to ROS (Aaseth et al., 2016) so that it can be neutralized.

The addition of glutathione in higher doses showed a decreasing motility graph. This is because adding glutathione too high can be toxic to spermatozoa cells. Carriço et al. (2023) stated that higher glutathione concentrations can damage the structure of axonema, which plays a vital role in the sperm motility process. Axonomea is found at the cell's tail and is composed of microtubules that rub against each other so spermatozoa can move. In addition, the uncontrolled addition of glutathione can alter the osmolarity of CEP diluents, thus impacting spermatozoa motility. Sperma motility is susceptible to being altered by the physical or chemical characteristics of the diluent used (Ansari et al., 2012).

Table 3 is the result of observing spermatozoa viability using the eosin-nigrosin staining. The characteristics of live spermatozoa are clear or unstained heads and dead spermatozoa have purplish red heads (Figure 1). According to Malinda et al. (2021), dead spermatozoa cells have damaged membranes, so eosin dye easily enters the cells.

Table 3 shows that sperm viability decreased from the initial viability. The decrease in sperm viability before freezing is due to a decreasing energy supply because the metabolic process of spermatozoa cells is still slow. This is emphasized by Manehat et al. (2021), who stated that a longer storage time causes a decrease in the percentage of sperm viability. The factor that causes the viability of spermatozoa to decline after freezing is the appearance of ROS, which can trigger lipid peroxidation

in the plasma membrane, resulting in damage to the spermatozoa membrane. According to Zhang et al. (2021), damaged spermatozoan membranes can cause spermatozoa viability to decrease.

Glutathione, which is one of the antioxidants, is done to reduce the presence of ROS during the freezing process. Adding various doses of glutathione in CEP diluent affected sperm viability in Balinese cattle before freezing and post-thawing ($P<0.05$). Agung et al. (2023) supported the idea that adding 0.2% glutathione can maintain the viability of Angus cattle. Adding 0.75 mM glutathione in CEP diluent resulted in the highest sperm viability before freezing (84.04%) and post-thawing (71.26%) compared to the other five treatments. A dose of 0.75 mM glutathione is the most effective dose in reducing the presence of ROS due to the composition of the amino acid cysteine. The amino acid cysteine, which makes up glutathione, contributes to donating electrons to ROS (Aaseth et al., 2016). Furthermore, the unpaired electrons will bind to the donor electrons of cysteine so that they become stable and non-reactive. Glutathione is an antioxidant that reduces free radicals through electron donation (Amorati and Valgimigli, 2018).

Table 4 shows the average of sperm plasma membrane integrity. Membrane integrity was evaluated by using the HOST method. Spermatozoa with coiled and curled tails indicate intact membranes, while straight-tailed spermatozoa indicate incomplete membranes (Figure 2). According to Prochowska et al. (2022), intact spermatozoa membranes exposed to hypoosmotic conditions cause the cells to swell because extracellular fluid enters the cells.

Table 4 shows that sperm plasma membrane integrity decreased during the storage and freezing process. One of the causative factors is the disruption of the osmotic pressure balance due to the dehydration process during freezing so that intracellular fluid moves out. Suhartati et al. (2020) emphasized that dehydrated cells can disrupt the balance of intracellular and extracellular osmotic pressure and impact physical and chemical cell damage. In addition, the freezing process can trigger lipid peroxidation, damaging the integrity of spermatozoa cell membranes (Yuslianti, 2018).

Based on Table 4 adding 0.75 mM glutathione in the CEP resulted in the highest percentage of sperm membrane integrity, before freezing (65.55±4.59%) and post-thawing (58.08±2.52%) compared to the other five treatments. The results were higher than those of the control treatment, which had no glutathione addition. Adding 0.75 mM in CEP affected the sperm plasma membrane integrity before freezing and post-thawing ($P<0.05$). This is in line with the research of Bebas et al. (2023) that glutathione in Andromed can maintain the integrity of the sperm membrane compared to control treatments.

Freezing processes in Balinese cattle spermatozoa cells can increase ROS (Silvestre et al., 2021), thereby damaging the structure of spermatozoa cells. Adding 0.75 mM glutathione was proven to maintain the integrity of the sperm membrane after freezing. The mechanism of glutathione in minimizing ROS is inactivating formed free radicals. Glutathione will react with free radicals to form glutathione radicals (GS•). This process occurs in a chain reaction until the two glutathione radicals react and form oxidized glutathione (Rizal and Herdis, 2010).

The addition of glutathione in higher doses showed a decreasing integrity of the sperm membrane graph. This suggests that adding glutathione in high doses impacted the integrity of the sperm membrane. Zhou et al. (2020) stated that high concentrations of glutathione could increase osmotic pressure in the diluent. Perumal et al. (2022) added that high antioxidants increase the fluidity of the plasma membrane of spermatozoa to above the optimal point. Thus, it can disturb the integrity of the spermatozoan membrane. Spermatozoa with damaged membrane integrity has impaired intermembrane substance exchange, resulting in disturbed metabolic process (Arvioges et al., 2021).

CONCLUSION

The addition of glutathione in CEP diluent affects the motility, viability, and membrane integrity of spermatozoa of Balinese cattle post-thawing. The best glutathione dose to maintain the quality of spermatozoa in Balinese cattle post-thawing was 0.75 mM, including motility of 55.30±2.95%, viability of 71.26±2.23%, and membrane integrity of 58.08± 2.52%. Further research is needed regarding the addition of glutathione in CEP diluents; it is necessary to pay attention to the osmolarity condition of the diluent.

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CONFLICT OF INTEREST

The authors declares no conflict of interest to disclosure.

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